[(+)(S)-VIII], α^{24}D +20.4 ± 0.5° (neat)[21] in ether solution. Distillation yielded 10.0 g. of (+)(S)-methyl β-phenyl-caproate, b.p. 139–142° (12 mm.), α^{24}D + 13.40 ± 0.05° (neat).

Anal. Calcd. for $C_{13}H_{18}O_2$: C, 75.7; H, 8.74. Found: C, 75.7; H, 8.92.

rac- and (+)(S)-1,1-Di-(*o*-anisyl)-3-phenylhexan-1-ol [*rac*-VI] and [(+)(S)-VI].—(+)(S)-Methyl β-phenylcaproate (α^{24}D +13.4°, neat), 3.6 g., was added dropwise to the reagent prepared from 10 g. of *o*-bromoanisole and 5 g. of magnesium. The slurry resulting was refluxed 6 hr. and dissolved by adding 100 ml. of water and 10 ml. of acetic acid. The ether layer was separated, dried (magnesium

(21) This rotation is higher than that reported by Levene and Marker[20] (α^{25}D +6.08°, neat); however, the acid is not optically pure.

sulfate) and evaporated. The residue crystallized from 15 ml. of benzene and 75 ml. of heptane, yielding a first crop of *rac*-VI which after recrystallization from benzene–heptane weighed 2.6 g., m.p. 98–99°, $[\alpha]^{23}$D ±0.1° (*c* 2, benzene).

Anal. Calcd. for $C_{26}H_{30}O_3$: C, 80.0; H, 7.73. Found: C, 79.7; H, 7.48.

Evaporation of the filtrate from the first crystallization of the *rac*-product yielded an oil which crystallized from 50 ml. of heptane and was recrystallized twice from ether–pentane to yield 0.75 g. of (+)(S)-VI, m.p. 93–94°, $[\alpha]^{23}$D +4.0 ± 0.3° (*c* 2.4, benzene). The infrared spectra of the *rac*., (+)- and (−)-alcohols were identical: $\lambda^{chloroform}_{max}$ 2.85, 3.40 and 6.23 μ.

Anal. Calcd. as above. Found: C, 80.4; H, 7.96.

[CONTRIBUTION FROM FACULTY OF PHARMACY, SCHOOL OF MEDICINE AND RESEARCH INSTITUTE FOR CATALYSIS AND DEPARTMENT OF CHEMISTRY, FACULTY OF SCIENCE, HOKKAIDO UNIVERSITY, SAPPORO, JAPAN]

Interaction between Synthetic ATP Analogs and Actomyosin Systems[1]

BY MORIO IKEHARA, EIKO OHTSUKA, SHOTARO KITAGAWA, KOICHI YAGI AND YUJI TONOMURA[2]

The following compounds were synthesized chemically as analogs of adenosine triphosphate (ATP); 6-methylamino-9-β-D-ribofuranosylpurine 5′-triphosphate (VIIIa), 6-dimethylamino-9-β-D-ribofuranosylpurine 5′-triphosphate (VIIIb), 3-β-D-ribofuranosyl-2-oxo-2,3-dihydropyrimidine 5′-triphosphate (X), 9-(4′-hydroxybutyl)-6-aminopurine 4′-triphosphate (VIb), 9-(3′-hydroxypropyl)-6-aminopurine 3′-triphosphate (VIa), 9-(2′-hydroxyethyl)-6-aminopurine 2′-triphosphate (VIc) and adenosine 5′-sulfatopyrophosphate (IX). The reactions of these analogs and deoxy-ATP with myosin B or myofibrils were investigated. The intensity of light scattered by myosin B was decreased by the addition of these compounds, except for X and XI, to the same extent as by ATP. Compound X was not hydrolyzed by myosin B. The velocities of hydrolysis of compounds other than X were of the same order of magnitude under various conditions as that of ATP. Myofibrils contracted after the addition of deoxy-ATP, VIIIa or VIIIb but not after the addition of X, VIb, VIa, VIc or IX. The initial rapid liberation of phosphate, which was shown on the hydrolysis of ATP, was not observed when VIc was the substrate. Inhibition by excess substrate was observed only in the hydrolysis of ATP and deoxy-ATP.

Introduction

The interactions between actomyosin systems and natural analogs of adenosine triphosphate (ATP), such as inosine triphosphate (ITP), uridine triphosphate (UTP), guanosine triphosphate and cytidine triphosphate, have been studied by several investigators[3-6] and especially by Blum[7] and Hasselbach.[8] These investigations have thrown some new light on the role of purine and pyrimidine bases in the contraction of muscle models.

Methods of synthesis of organic triphosphate compounds have been developed by many investigators, especially by Todd[9,10] and Khorana[11] and their collaborators, and recently by Hasselbach[12] and Kessler.[13] Therefore, it might be expected

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(8) W. Hasselbach, *Biochim. et Biophys. Acta*, **20**, 355 (1956).

(9) J. Baddiley, A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 582 (1949).

(10) V. M. Clark, G. W. Kirby and A. R. Todd, *ibid.*, 1497 (1957).

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

(12) W. Hasselbach, *Acta biol. med. germ.*, **2**, 13 (1959).

(13) D. Kessler, B. Moss and R. W. Chambers, *Biochim. et Biophys. Acta*, **38**, 549 (1960).

that the roles of the three parts of the ATP molecule (adenine base, ribose and triphosphate) in muscle contraction might be revealed by synthesizing drastically modified analogs of ATP and investigating their reactions with actomyosin systems at three levels, *i.e.*, myosin B solution at a high ionic strength, its suspension at a low ionic strength and isolated myofibrils. However, to the authors' knowledge, only two reports have been published in this field. They concerned the use of diacetyl-ATP[8] and adenyl methylenediphosphonate[14] as modified compounds of ATP.

The present authors have synthesized the following ATP analogs; 6-methylamino-9-β-D-ribofuranosylpurine 5′-triphosphate (VIIIa), 6-dimethyl-amino-9-β-D-ribofuranosylpurine 5′-triphosphate (VIIIb), 3-β-D-ribofuranosyl-2-oxo-2,3-dihydropyrimidine 5′-triphosphate (X), 9-(4′-hydroxybutyl)-6-aminopurine 4′-triphosphate (VIb), 9-(3′-hydroxypropyl)-6-aminopurine 3′-triphosphate (VIa), 9-(2′-hydroxyethyl)-6-aminopurine 2′-triphosphate (VIc) and adenosine 5′-sulfatopyrophosphate (IX). It is the purpose of this report to describe the methods of synthesis of these analogs and the properties of their reactions with actomyosin systems and also to clarify the role of the ATP molecule in muscle contraction.

Results

The velocity of liberation of phosphate and the decrease in light-scattering by myosin B in the presence of ATP or its analog fluctuated considerably

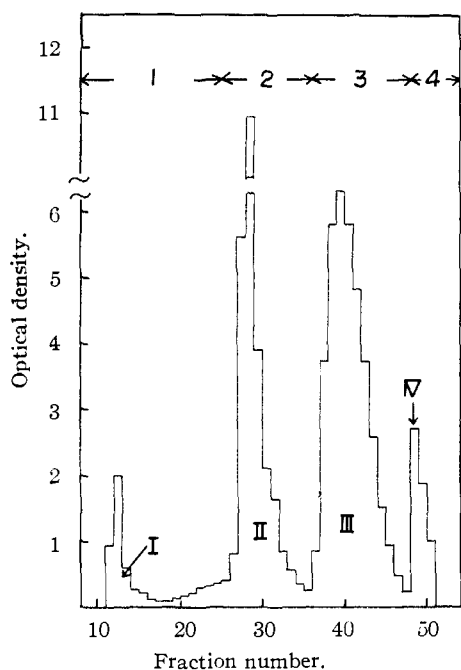(14) C. Moos, N. R. Alpert and T. C. Myers, *Arch. Biochem. Biophys.*, **88**, 183 (1960).

Fig. 1.—Elution pattern of chromatography of 9-(3'-hydroxypropyl)-6-aminopurine 3'-phosphate. Amberlite IRA 400. Column 1.8 × 7 cm. The first, second, third and fourth eluting solutions are 0.003 N HCl, 0.003 N HCl + 0.05 M LiCl, 0.003 N HCl + 0.1 M LiCl and 2 N HCl, respectively. Nucleosidephosphate concentration expressed as optical density at 260 mμ. The fractions I, II, III and IV are the mono-, di-, tri- and higher phosphates, respectively.

from one myosin B preparation to another. Therefore, in this report, the ratio of the value obtained with each analog to that of ATP for one myosin B preparation is used as an expression of the activity. In Table I are shown the mean values of the velocities of ATPase at the steady state of four myosin B preparations in three ionic media used for ATPase assay.

TABLE I

MEAN VELOCITY OF MYOSIN B-ATPASE EXPERIMENTS AT pH 7.0 AND 20°

| KCl	0.6 M		0.075 M
Divalent cation Concentration	7 mM Ca^{++}	1 mM Mg^{++}	2 mM Mg^{++}
of ATP	1 mM	1 mM	0.1 mM
Velocity^a	2.7	0.28	6.1

^{a} Values expressed as μ moles phosphate/g./sec.

Synthesis of ATP Analog.—9-(2'-Hydroxyethyl)-6-aminopurine 2'-triphosphate (VIc) was synthesized by the method described in a previous paper.[15] The modification of the ribose part of ATP was further extended to the synthesis of 9-(3'-hydroxypropyl)-6-aminopurine 3'-triphosphate (VIa) and 9-(4'-hydroxybutyl)-6-aminopurine 4'-triphosphate (VIb). 4,6-Dichloro-5-aminopyrimidine (I) was converted to 6-chloropurines (IIIa,b) via 4-alkanolamino-5-amino-6-chloropyrimidines (IIa,b) by the successive dechloroamination and cyclization with acetic anhydride and ethylorthoformate in an

(15) M. Ikehara and E. Ohtsuka, Chem. & Pharm. Bull. (Tokyo), 9, 27 (1961).

over-all yield of 60 %. The amination of IIIa and IIIb led to an unambiguous synthesis of 9-(ω-hydroxy-alkyl)-adenines (IVa,b), which were phosphorylated by the use of polyphosphoric acid[16] to their monophosphates (Va, b). The monophosphates were caused to react further with orthophosphoric acid and excess dicyclohexylcarbodiimide (DCC).[11] As a typical example of the chromatographies of the analogs, the elution pattern of ion-exchange chromatography of VIa is shown in Fig. 1. The third peak was collected, neutralized and lyophilized. The triphosphate was isolated from lithium chloride by extraction with absolute methanol. The product was analyzed by means of paper chromatography and elementary analysis.

6-Methylamino-9-β-D-ribofuranosylpurine 5'-triphosphate (VIIIa) and 6-dimethylamino-9-β-D-ribofuranosylpurine 5'-triphosphate (VIIIb), in which the 6-amino group of ATP was methylated, were synthesized from 6-methylamino-9-β-D-ribofuranosylpurine 5'-monophosphate (VIIa)[17] and 6-dimethylamino-9-β-D-ribofuranosylpurine 5'-monophosphate (VIIb)[17] by treatment with 85 % orthophosphoric acid in the presence of DCC.[11] The triphosphate was isolated by ion-exchange chromatography and purified as described in the Experimental.

Adenosine 5'-sulfatopyrophosphate (IX) was synthesized from adenosine 5'-diphosphate (ADP) by the reaction with pyridine–sulfur trioxide in a manner similar to the method reported by Baddiley[18] for the synthesis of adenosine 3'-phosphate
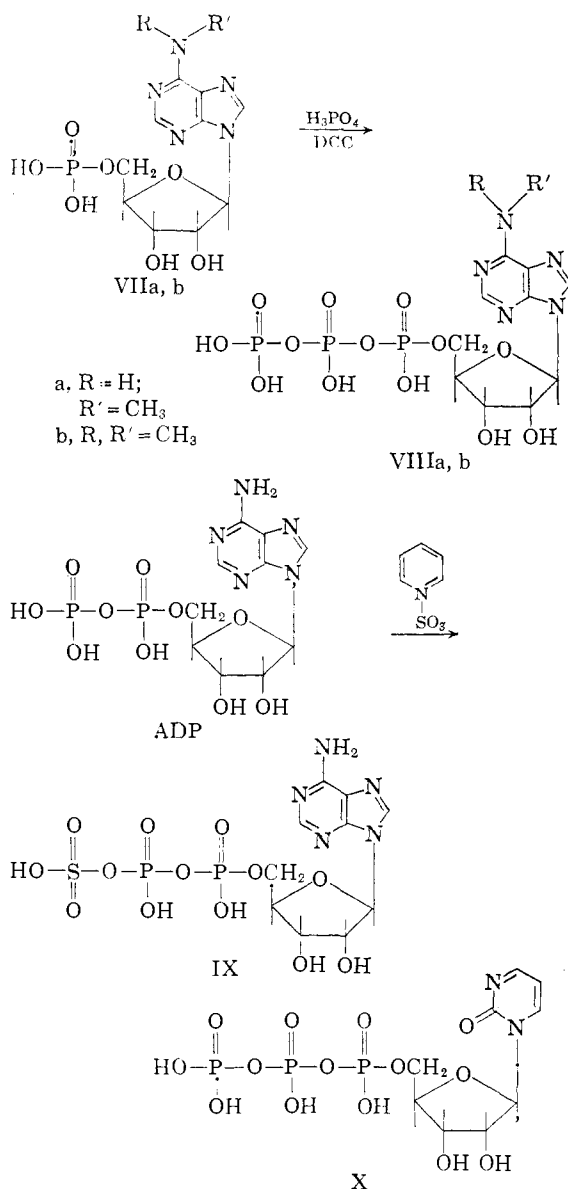

a, $n = 3$
b, $n = 4$
c, $n = 2$
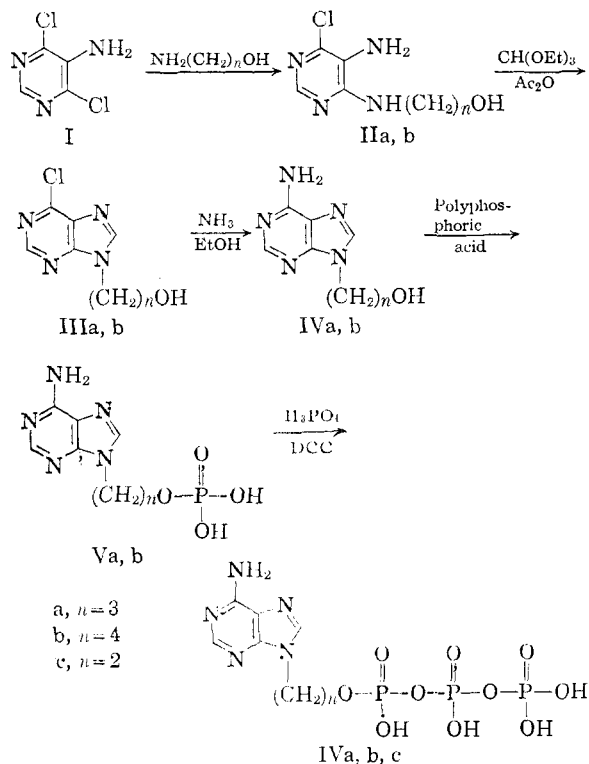
(16) R. H. Hall and H. G. Khorana, J. Am. Chem. Soc., 77, 1871 (1955).

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5′-sulfatophosphate. The structure of the resulting product was confirmed by the presence of labile sulfate and ADP (see Experimental).

The main contaminant of these synthesized specimens of ATP analogs was lithium chloride, of which the concentration in the biochemical reaction medium was too low to produce any appreciable effect on the actomyosin system.

VIIa, b

a, R = H;
R′ = CH₃
b, R, R′ = CH₃

VIIIa, b

ADP

IX

X

Fig. 2.—Decrease in intensity of light scattered by myosin B on addition of ATP analogs. Myosin B no. 2. 0.6 M KCl, 1 mM Mg^{++}, pH 7.0, 15°: O, 0.5 mM ATP; △, 0.36 mM VIIIb; X, 0.4 mM VIa. The arrow indicates addition of ATP or ATP analog.

amount of the analogs to myosin B solution. In columns 5 and 6 of Table II are summarized the results of light-scattering experiments, together with the results obtained with ITP and UTP by Blum.[7] The maximum decrease in light-scattering by the addition of the analogs, except for compounds X and IX, was the same as that induced by ATP. In the case of IX, both the maximum value and the velocity of drop were considerably less than those for ATP. It was shown by paper chromatography[23] that IX was very unstable and was hydrolyzed to ADP and sulfate in the reaction medium, even in the absence of myosin B. Therefore, the drop in light-scattering caused by the addition of IX may have been due to ATP formed from ADP by myokinase present as a contaminant in the myosin B.

Typical examples of the relation between the degree of change of light-scattering (Δ/Δ_c) and the concentration of the analog are illustrated in Fig. 3. Here Δ and Δ_c are defined by

$$\Delta = I_0 - I_d$$
$$\Delta_c = I_0 - I_\infty$$

where I_0 and I_d are the intensities of light-scattering before and after the addition of some amount of the analog respectively and I_∞ is the intensity after the addition of a sufficient amount of the analog. The strength of binding as judged by the change in light-scattering was in the decreasing order of deoxy-ATP $\geqq$ ATP $\geqq$ VIIIb > VIc > UTP $\geqq$ VIb $\geqq$ VIa $\geqq$ ITP (Column 6 of Table II). Here the positions of UTP and ITP were fixed by the results of Blum.[7]

Decrease in Light-scattering.—As reported,[19–22] at a high ionic strength the molecular shape of the main components of myosin B is elongated and the intensity of light scattered by myosin B is decreased by the addition of ATP or pyrophosphate. In Fig. 2 are given typical examples of the decrease in light-scattering after the addition of a sufficient

(19) J. J. Blum and M. F. Morales, Arch. Biochem. Biophys., 43, 208 (1953).

(20) M. F. Gellert, P. H. v n Hippel, H. K. Schachman and M. F. Morales, J. Am. Chem. Soc., 81. 1384 (1959).

(21) T. Nihei and Y. Tonom i a, J. Biochem., 46, 1355 (1959).

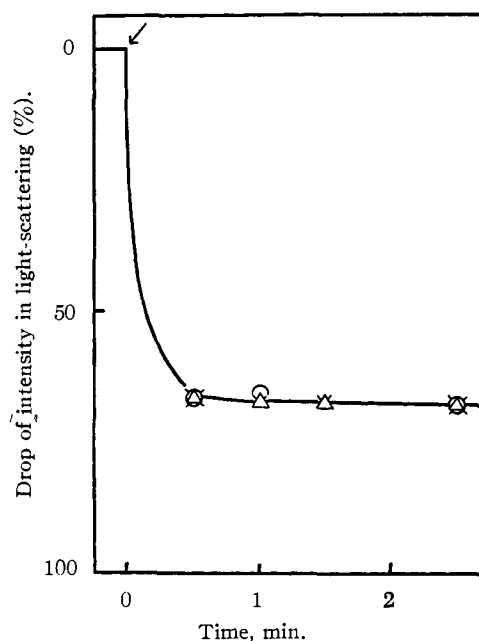(22) F. Morita and Y Tonu ura, J. Am. Chem. Soc., 82, 5172 (1960).

(23) R_f of IX in the solvent S′ (n-PrOH:NH₄OH:H₂O = 6:3:3) was 0.43. After the incubation of IX with myosin B and/or Ca^{++} in 0.6 M KCl, R_f changed to 0.34–0.37, which was almost identical to that of ADP, 0.34 (unpublished results by M. Ikehara and E. Ohtsuka)

TABLE II

INTERACTION BETWEEN ATP ANALOGS AND ACTOMYOSIN SYSTEMS

Comp.	Base	Sugar	Phosphate	Decrease in light-scattering of myosin B[a] Max. decrease	Ratio of concentrations of analogs for decrease of 50% of max.	Velocity of hydrolysis by myosin B[c] Concn., mM	—0.6 M KCl— 7 mM Ca++	1 mM Mg++	0.075 M KCl 2 mM Mg++	Contraction of myofibrils[e]	Substrate inhibition of hydrolysis[g]
ATP	6-NH2-purine	D-ribose	–P–P–P	100	1	1	100	100		+	+
						0.1			100		
VIIa	6-NHCH3-purine	D-ribose	–P–P–P	89		0.92	180	160	100	+	–
VIIIb	6-N(CH3)2-purine	D-ribose	–P–P–P	100	1.2	1.2	70	105	10	+	–
ITP	6-OH-purine	D-ribose	–P–P–P		24[b]		300[b]		50[d]	+	–
UTP	2,6-(OH)2-pyrimidine	D-ribose	–P–P–P		4.4[b]		200[b]		85[d]	+	–
X	2-OH-pyrimidine	D-ribose	–P–P–P	15		0.88	0	28	5	–	–
Diacetyl-ATP	6-NH2-purine	2',3'-Diacetyl-D-ribose	–P–P–P						120[d]	+[d]	+[d]
Deoxy-ATP	6-NH2-purine	Deoxy-D-ribose	–P–P–P	100	0.8	.9	110	190		+[f]	+
						.09			110		
VIb	6-NH2-purine	–(CH2)4-O–	–P–P–P	100	4.5	.92	75	410	45	–	–
VIa	6-NH2-purine	–(CH2)3-O–	–P–P–P	100	5.8	.73	100	300	30	–	–
VIc	6-NH2-purine	–(CH2)2-O–	–P–P–P	97	2.7	.88	65	640	40	–	–
IX	6-NH2-purine	D-ribose	–P–P–S	(57.5)							

[a] Experiments at 0.6 M KCl, 1 mM Mg++, pH 7.0 and room temperature. [b] J. J. Blum, Arch. Biochem. Biophys., 55, 486 (1955). [c] Experiments at pH 7.0 and 20°. [d] W. Hasselbach, Biochim. et Biophys. Acta, 20, 355 (1956). [e] Experiments at 0.02 M KCl, 2.6 mM Mg++, about 2 mM analog, pH 7.0 and room temperature. [f] Concentration of deoxy-ATP was about 0.2 mM. [g] Experiments at 0.075 M KCl, 2 mM Mg++, pH 7.0 and 20°.

Velocity of Phosphate-liberation at Steady State.

Since the rate of liberation of phosphate from the ATP-myosin B system was, in an earlier stage, several times higher than the constant value ulti-
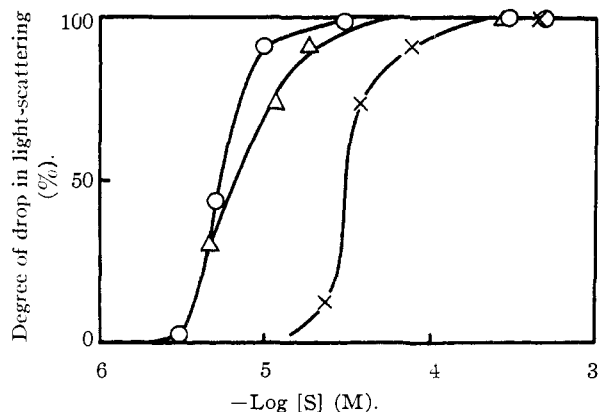

Fig. 3.—Relation between degree of change of light-scattering and concentration of ATP analogs. Myosin B no. 2. 0.6 M KCl, 1 mM Mg++, pH 7.0, 15°: O, ATP; △, VIIIb; ✕, VIa.

mately attained at the steady state,[24-26] at first the rate of liberation of phosphate from the organic triphosphate-myosin B system was measured at the steady state.

As is well known,[27,28] myosin B ATPase is activated by Ca++ and inhibited by Mg++ at a high ionic strength but is activated both by Ca++ and Mg++ at a low ionic strength. In columns 7–10 of Table II are summarized the velocities of phosphate-liberation in the presence of Ca++ or Mg++ at a high ionic strength and in the presence of Mg++ at a low ionic strength, along with the data on ITP, UTP and diacetyl-ATP reported by Blum[7] and Hasselbach.[8] To avoid inhibition of hydrolysis by excess substrate (see below), a concentration of about 0.1 mM was used, particularly when ATP or deoxy-ATP was used as the substrate in the presence of 0.075 M KCl and 2 mM Mg++.

As clearly shown in Table II, the velocities of hydrolysis of ATP analogs depend more distinctly on their chemical structures than on light-scattering. The dependence varied with the ionic condition of the medium. Compound VIIIa was hydrolyzed at a higher rate than ATP in 0.6 M KCl and at the same rate as ATP in 0.075 M KCl. Compound VIIIb was hydrolyzed at a rate similar to that of ATP in 0.6 M KCl and at a much lower rate than ATP in 0.075 M KCl. Compound X was scarcely hydrolyzed under all the experimental conditions. The rate of hydrolysis of deoxy-ATP was similar to or higher than that of ATP under suitably varied conditions. The rates of hydrolysis of VIb, VIa and VIc were similar to that of ATP in the presence of 0.6 M KCl and 7 mM Ca++, and higher than that of ATP in the presence of 0.6 M KCl and 1 mM Mg++; but in the presence of 0.075 M KCl and 2 mM Mg++ they were only 30–45 % of that of ATP. Generally speaking, when the structure of the base or the ribose part was modified, the velocity of phosphate-liberation did not change markedly at a high ionic strength and in the presence of Ca++, though it decreased at a low ionic strength

(24) A. Weber and W. Hasselbach, Biochim. et Biophys. Acta, 15, 237 (1954).
(25) Y. Tonomura and S. Kitagawa, ibid., 27, 15 (1957).
(26) Y. Tonomura and S. Kitagawa, ibid., 40, 135 (1960).
(27) I. Banga, Stud. Inst. Med. Chem. Univ. Szeged., 1, 27 (1941).
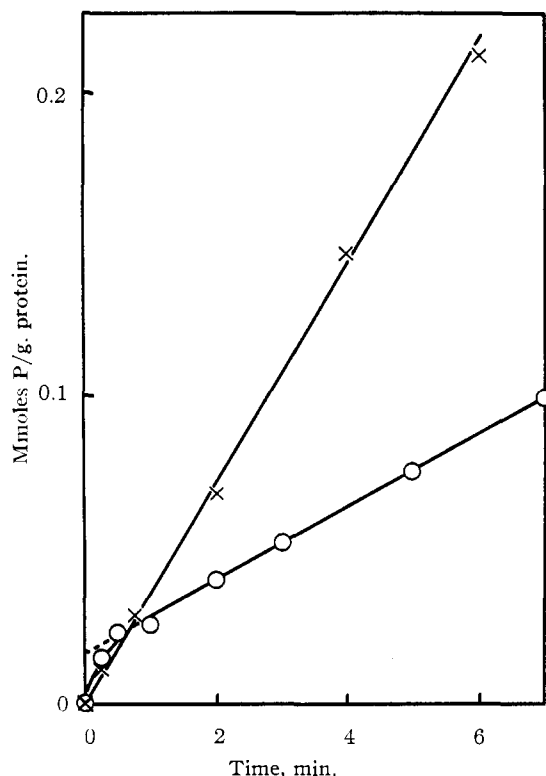(28) T. Nihei and Y. Tonomura, J. Biochem., 46, 305 (1959).

Fig. 4.—Initial phase of phosphate-liberation from ATP and VIc. Myosin B no. 3. 0.6 M KCl, 1 mM Mg^{++}, pH 7.0, 20°: ○, 0.9 mM ATP; ✕, 0.88 mM VIC.

and in the presence of Mg^{++} as the structure of the analog was altered from that of ATP.

It has been demonstrated[6,29] that in 0.6 M KCl, ethylenediamine tetraacetic acid activates myosin B ATPase strongly and UTPase slightly but does not activate ITPase. At pH 8.0 and 20° and in 0.6 M KCl, the addition of 2 mM of ethylenediamine tetraacetic acid reduced the rates of hydrolysis of VIa and VIb to about 20 % of the control rates, though it led to an approximately 5 fold increase in the ATPase activity of the myosin B used in this study.

Initial, Rapid Liberation of Phosphate.—The initial rapid liberation of phosphate[24-26] depended more strictly on the chemical structure of the analog than did the steady liberation of phosphate. As illustrated in Fig. 4, the initial phase of phosphate-liberation was not observed with IVc, in which the ribose part of ATP was replaced by –CH$_2$–CH$_2$O–.

Contraction of Myofibrils.—In column 11 of Table II are summarized the results on the contraction of isolated myofibrils resulting from the addition of ATP analogs. As described in the preceding sections, the analogs, except for X and IX, decreased the intensity of light scattered by myosin B to the same extent as ATP did and were hydrolyzed by myosin B at rates comparable to that for ATP. As expected from these results, the compounds X and IX were inert by the criterion of contraction of myofibrils. But the more remarkable fact was that myofibrils did not contract on the addition of VIb, VIa or VIc. It should be noted

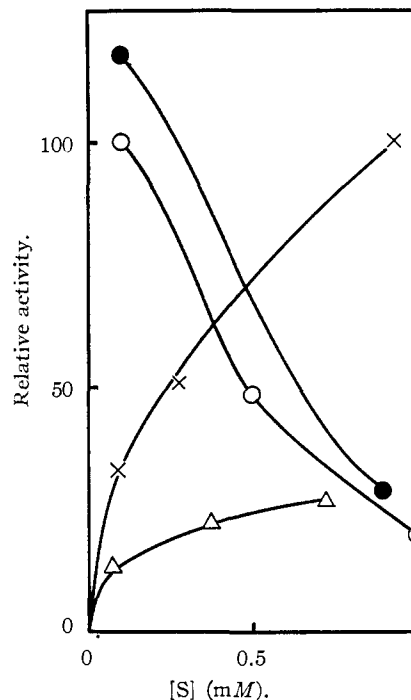(29) W. Hasselbach, *Biochim. et Biophys. Acta*, **20**, 440 (1956).

Fig. 5.—Dependence of velocity of hydrolysis on concentration of substrate at low ionic strength and in presence of Mg^{++}. 0.075 M KCl, 2 mM Mg^{++}, pH 7.0, 20°: ○, ATP, myosin B no. 2; ●, deoxy-ATP, myosin B no. 3; ✕, VIIIa, myosin B no. 1; △, VIa, myosin B no. 2.

that at a low ionic strength and in the presence of Mg^{++} the steady velocities of hydrolysis of VIb, VIa and VIc were much higher than that of VIIIb which caused contraction of myofibrils and that the initial, rapid liberation of phosphate did not appear with VIc.

Inhibition by Excess Substrate.—At a low ionic strength and in the presence of Mg^{++}, the velocity of myosin B-ATPase falls as the concentration of ATP is increased, when the concentration is higher than 0.1 mM.[30,31] As already indicated by several investigators,[31-33] this phenomenon, the inhibition by excess substrate, is closely connected with the relaxation of muscle models.

As shown in column 12 of Table II and Fig. 5, the inhibition by excess substrate complied most strictly with the chemical structures of the analogs. The inhibition could be observed only with ATP, diacetyl-ATP[8] and deoxy-ATP and not with VIb, VIa and VIc (which had -(CH$_2$)n-CH$_2$O- in place of the ribose of ATP and did not cause contraction of myofibrils) or with VIIIa and VIIIb which still had the ribose, but in which 6-NH$_2$ in the adenine base was methylated.

Discussion

As already described, the intensity of light scattered by myosin B was decreased to the same extent by various ATP analogs, except for compounds X and IX, and the same analogs were hydrolyzed by

(30) W. Hasselbach, *Z. Naturforsch.*, **7b**, 163 (1952).

(31) Y. Tonomura and J. Yoshimura, *Arch. Biochem. Biophys.*, **90**, 73 (1960).

(32) S. S. Spicer, *J. Biol. Chem.*, **199**, 289 (1952).

(33) H. H. Weber and H. Portzehl, *Progress in Biophys.*, **4**, 60 (1954).

myosin B at rates similar to that for ATP. The initial rapid liberation of phosphate ("initial burst") and the contraction of myofibrils were observed only with the analogs in which the ribosyl group was involved. Moreover, the hydrolysis of the analog by myosin B was not inhibited by excess substrate unless the analog had an intact adenine base in addition to the ribose ring. It should be noted that the compounds VIb, VIa and VIc could not contract myofibrils and that VIc did not show the initial burst, even though these compounds did decrease the intensity of light-scattering by myosin B solution and were hydrolyzed by myosin B.

It is reasonably assumed[34] that Mg++ or Ca++ mediates the binding between the terminal phosphate of the nucleoside triphosphate and the myosin molecule. The result that IX does not change the intensity of light-scattering by myosin B may be attributable to unadaptability of the terminal sulfate to this binding. Blum[7] previously indicated the importance of the amino group at the position 6 of adenine to the binding of ATP with myosin. This explanation is supported by the fact that the compound X, which lacks the 6-amino group, does not interact with myosin B, though cytidine triphosphate interacts with actomyosin systems in a manner similar to that of ATP.[7,8]

The mechanism involving the necessity for the ribosyl group in the nucleoside triphosphate for the contraction of myofibrils and for the initial burst is not yet clear. However, certain possibilities are worth mentioning. In the first place, it may depend on the distance between the phosphorylated O atom and the C–N[9] bond. This distance in ATP is 1.4 Å longer than that in VIa but 0.1–0.2 Å shorter than that in VIb.[35,36] Therefore this distance cannot be the deciding factor in the inability of VIb to cause contraction of myofibrils. Secondly, it may be supposed that a binding of the ATP analog to myosin at the ribose part is necessary for muscle contraction. However, at least the hydroxyl group of the ribose cannot be necessary for the binding of ATP with myosin, because diacetyl- and deoxy-ATP can interact with myosin B and cause contraction of myofibrils. Furthermore, ATPase was not inhibited by the addition of 0.1 M of D-ribose.[37] The third and most probable explanation involves the rigidity of the structure of ribose. The C–C bonds of –(CH$_2$)n– in VIb, VIa and VIc permit free rotation, so that when the compounds bind to the active site of myosin in the correct configuration, there will be a loss of the configurational entropy. Hence the combination with myosin necessary for muscle contraction may be improbable in the case of VIb, VIa and VIc.

Experimental

Myosin B solutions were prepared from minced rabbit skeletal muscle as described in one of the previous papers.[22] Myofibrils were isolated from rabbit back muscle and pre-

(34) S. Kitagawa and Y. Tonomura, *J. Research Inst. Catalysis* (Hokkaido U.), **8**, 91 (1960).

(35) G. W. Wheland, "The Theory of Resonance and its Application to Organic Chemistry," John Wiley and Sons, Inc., New York, N. Y., London, 1947.

(36) M. H. F. Wilkins, "Nucleoproteins," Solvay Int. Inst. Chem., 11th Chem. Conference, Interscience Publishers, Inc., New York, N. Y., 1959, p. 45.

(37) N. Azuma, unpublished observations.

pared by the method of Perry[38] with slight modifications.[39]

Measurements of the scattering of light were carried out at an angle of 90° from the incident beam by the method described previously.[22] The ATP analog was added to a 0.6 M KCl solution of myosin B in the presence of 1 mM Mg++ and at pH 7.0 and room temperature.

The rate of hydrolysis of the ATP analog was determined at 20° and pH 7.0, using 7 mM Ca++ or 1 mM Mg++ as a modifier in 0.6 M KCl or 2 mM Mg++ in 0.075 M KCl. The concentration of the analog was about 1 mM, unless otherwise stated. The reaction was stopped by trichloroacetic acid at measured time intervals, and inorganic phosphate was determined by the Martin–Doty method.

The contraction of myofibrils was observed with a phase-contrast microscope (Olympus Model E) with 800 fold magnification. The criterion for contraction was formation of the characteristic "contraction band." The incubation mixture had the following composition: about 1.5 mg./ml. of myofibrils, 2.6 mM Mg++, 0.02 M or 0.1 M KCl and about 2 mM of the substrate, except for deoxy-ATP. 0.2 mM of deoxy-ATP was used, for contraction was not clearly observed at 2 mM.

The protein content was determined by the Biuret method or by multiplying the nitrogen content determined by the micro-Kjeldahl method by 6.

ATP and deoxy-ATP were purchased from Sigma Co.

Syntheses of ATP Analogs. Paper Chromatography.— Toyo Filter Paper No. 51-A was used. Solvent A, IsoPrOH: 1 % (NH$_4$)$_2$SO$_4$ aq. = 2:1; A' = 3:2; B, ButOH:H$_2$O = 86:14; H, saturated (NH$_4$)$_2$SO$_4$:H$_2$O:IsoPrOH = 79:19:2; H', H$_2$O was replaced by 0.1 M AcONH$_4$; S, n-PrOH:NH$_4$-OH:H$_2$O = 6:3:1; S' = 6:3:3. All chromatograms, except for the case of the solvent B, were made by the descending technique.

9-(2'-Hydroxyethyl)-6-aminopurine 2'-triphosphate (VIc)[15] and 3-β-D-ribofuranosyl-2-oxo-2,3-dihydropyrimidine 5'-triphosphate (X)[40] were synthesized as described in previous papers.

9-(3'-Hydroxypropyl)-6-aminopurine 3'-Triphosphate (VIa).—(i) 4-(3'-Hydroxypropyl)-5-amino-6-chloropyrimidine (IIa).—5.1 g. of 4,6-dichloro-5-aminopyrimidine[41] was dissolved in dioxane (30 ml.) and added to a solution of 4.7 g. of 3-amino-1-propanol in 15 ml. of dioxane. After 18 hours' reflux, dioxane was evaporated, the residue was washed with ice-water and recrystallized from water. 5.1 g. of needles, m.p. 86–87°, was obtained; yield, 81%.

Anal. Calcd. for C$_7$H$_{11}$N$_4$OCl·H$_2$O: C, 38.09; H, 5.89. Found: C, 38.60; H, 5.69.

(ii) 9-(3'-Hydroxypropyl)-6-aminopurine (IVa).—A mixture of 4.8 g. of IIa, 20 ml. of ethylorthoformate and 20 ml. of acetic anhydride was warmed gently to boiling and then refluxed for 3 hr. at 130°. Volatile materials were removed by codistillation with ethanol. The vitreous oil thus obtained had λ_{max}^{EtOH} 265 mμ and showed cyclization to the purine (IIIa), which was used for further reaction without purification. IIIa was heated at 120° for 18 hr. in an autoclave with 200 ml. of EtOH saturated with dry ammonia at 0°. After cooling, ammonium chloride was removed from the precipitated organic material by decantation. The organic material was recrystallized from EtOH, m.p. 204–206° (1.2 g.) From the above decantate, 1.7 g. of IVa was recovered; yield, 68% (m.p. reported by Lister and Timmis[42] was 194–198°).

Anal. Calcd. for C$_8$H$_{11}$ON$_5$: C, 49.74; H, 5.76. Found: C, 49.77; H, 5.51.

Paper chromatography: R_f, 0.32 (solvent B), 0.74 (solvent S'). ultraviolet spectrum: $\lambda_m^{n\Pi}$, 260 mμ (molar extinction coefficient, ϵ = 13.2 × 10^3), λ_{min}^{nH} 229 mμ; $\lambda_{max}^{0.1 N HCl}$ 259 mμ (12.4 × 10^3), $\lambda_{min}^{0.1 N HCl}$ 229 mμ; $\lambda_{max}^{0.1 N NaOH}$ 259 mμ (12.6 × 10^3), $\lambda_{min}^{0.1 N NaOH}$ 225 mμ.

(iii) 9-(3'-Hydroxypropyl)-6-aminopurine 3'-monophosphate (Va).—1.0 g. of IVa was added to a mixture of 4.7 g. of 85 % phosphoric acid and 3.6 g. of phosphorus pentoxide.[16] Even after 4 hr. reaction at 60°, a considerable amount of the starting material remained unchanged. This was separated by decantation and was caused to react separately for 6

(38) S. V. Perry and A. Corsi, *Biochem. J.*, **68**, 5 (1958).

(39) K. Yagi and L. Noda, *Biochim. Biophys. Acta*, **43**, 149 (1960).

(40) M. Ikehara, *Chem. & Pharm. Bull. (Tokyo)*, **8**, 303, 836 (1960).

(41) D. J. Brown, *J. Appl. Chem.*, **4**, 72 (1954).

(42) J. H. Lister and G. M. Timmis, *J. Chem. Soc.*, 327 (1960).

more hr. with a half amount of the reagents. Both these reaction mixtures were combined. To hydrolyze polyphosphates, the mixture was heated at 100° for 20 minutes after addition of 30 ml. of water, and then pH was adjusted to 6.5 with hot, saturated barium hydroxide. The precipitate was filtered and washed with hot water (3 × 100 ml.). The filtrate and the washings were combined and concentrated to ca. 30 ml. under reduced pressure. The resulting white crystals were collected (0.4 g.) by filtration, and the pH of the mother liquor was adjusted to 7.1 with barium hydroxide. The precipitate was collected by centrifugation. Both the precipitates contained no inorganic phosphate and showed the same R_f (0.33, solvent S'); yield, 0.2 g. The mother liquor gave a spot of IVa (R_f 0.74) on the paper chromatogram. Addition of 2 volumes of EtOH to this liquor gave a precipitate of barium salt of Va, which was washed with EtOH and then with ether, and redissolved in 150 ml. of water. As this solution contained 1 mmole of Va and gave only one spot on the paper chromatogram by the two solvent systems (R_f 0.49, solvent A'; 0.19, solvent H'), it was used in the successive synthesis without isolation of Va.

(iv) 9-(3'-Hydroxypropyl)-6-aminopurine 3'-Triphosphate (VIa).[11]—The water solution of 1 mmole of Va was neutralyzed by 1 N sulfuric acid (ca. 2 ml.) in the presence of Na-rhodizonate indicator.[43] The barium sulfate precipitated was removed by centrifugation. The washings and the filtrate were combined, mixed with a small excess of tri-n-butylamine and evaporated under reduced pressure. 1.2 g. of the residue was dissolved in 1.2 g. of 85 % phosphoric acid, 5 ml. of tri-n-butylamine and 20 ml. of pyridine. The slightly turbid solution was caused to react with 12 g. of dicyclohexyl carbodiimide (DCC) for 36 hr. at 20°.[11] Dicyclohexylurea, thus produced, was removed by filtration, washed with water, and the filtrate and the washings were adjusted to 50 ml. After ether extraction, this solution was readjusted to 200 ml. (pH 6.5). 10 g. of activated charcoal[18] was added to the solution portionwise with stirring. The charcoal was collected and 2 g. of charcoal was added to the filtrate. The combined charcoal samples were washed with water until no phosphate was detected and finally eluted with 50 % EtOH–water containing 2 % ammonia (recovery of this stage was 61 %). The effluent was evaporated to 50 ml. in a rotary evaporator at less than 25° (TOD$_{280}$ 14700, TOD$_{280}$ 2300)[44] and applied to the top of a column of Amberlite IRA 400 (Cl⁻-form, 1.8 × 7 cm.). After washing with water, monophosphate (TOD$_{260}$ 465, 5.6 %), diphosphate (TOD$_{260}$ 3060, 37 %), triphosphate (TOD$_{260}$ 4400, 53 %) and higher phosphates (TOD$_{260}$ 350, 4.2%) were eluted with 0.003 N HCl, 0.003 N HCl + 0.05 N LiCl, 0.003 N HCl + 0.1 N LiCl and 2 N HCl, respectively (Fig. 1). The triphosphate fraction was collected, adjusted to pH 7.0 with 2 N LiOH, concentrated under reduced pressure and freezedried. Lithium chloride was extracted with absolute MeOH.[46] The remaining white powder was dissolved in water and reprecipitated by the addition of EtOH. 74 mg. of VIa was obtained; yield, 14 %.

Anal. Calcd. for C$_8$H$_{10}$O$_{10}$N$_5$P$_3$Li$_2$·8H$_2$O: total P, 15.5; labile P, 10.3. Found: total P, 15.0; labile P, 9.9.

Purity on a weight basis as estimated spectrophotometrically 84% (ϵ of 6-amino-9-propanolpurine = 14.6 × 10³), estimated from P analysis 75 %. The ratio of base: total P: labile P = 1.0:2.7:1.7 (theoretical; 1:3:2). Paper chromatography gave only one spot in the two solvent systems: R_f 0.43 (solvent A'), 0.33 (solvent H').

9-(4'-Hydroxybutyl)-6-aminopurine 4'-Triphosphate (VIb). (i) 4-(4'-Hydroxybutyl)-5-amino-6-chloropyrimidine (IIb).—2.8 g. of 4-amino-1-butanol[47] and 1.1 g. of triethylamine dissolved in 10 ml. of dioxane were added into a solution of 3.5 g. of 4,6-dichloro-5-aminopyridine[45] and refluxed for 15 hr. Evaporation of dioxane and cooling gave a solid material, which was washed with a small amount of water

(43) A. M. Michelson, J. Chem. Soc., 1957 (1958).

(44) As defined by Chambers and Khorana,[45] TOD$_{260}$ stands for the observed optical density at 260 mμ multiplied by the number of ml. of the solution.

(45) R. W. Chambers and H. G. Khorana, J. Am. Chem. Soc., 80, 3749 (1958).

(46) R. W. Chambers, ibid., 81, 3032 (1959).

(47) German Patent 730,237 (1942); Chemical Abstracts, 38, 381 (1944).

and recrystallized from water. 2.4 g. of needles, m.p. 136–138°, was obtained; yield, 52%.

Anal. Calcd. for C$_8$H$_{13}$ON$_4$Cl: C, 44.34; H, 6.00. Found: C, 44.04; H, 6.30.

(ii) 9-(4'-Hydroxybutyl)-6-aminopurine (IVb).—2 g. of IIb was refluxed in 10 ml. of acetic anhydride and 10 ml. of ethylorthoformate for 3 hr. Volatile material was removed by distillation and codistillation with anhydrous alcohol. The vitreous material obtained showed λ_{max} at 260 mμ, indicating the conversion from IIb (λ_{max}, 287 mμ and 260 mμ) to IIIb. It was heated at 100–120° for 14 hr. in an autoclave with 100 ml. of ethyl alcohol saturated with ammonia at 0°. The solvent was evaporated and the residue was recrystallized from anhydrous ethanol, m.p. 196–197°; yield, 1.2 g. (63 %).

Anal. Calcd. for C$_9$H$_{13}$ON$_5$: C, 52.14; H, 6.32; N, 33.81. Found: C, 51.77; H, 6.60; N, 33.74. R_f: 0.36 (solvent B). $\lambda_{max}^{pH 7}$ 261 mμ (ϵ = 14.7 × 10³), $\lambda_{min}^{pH 7}$ 231 mμ; $\lambda_{max}^{0.1 N HCl}$ 258 mμ (11.9 × 10³), $\lambda_{min}^{0.1 N HCl}$ 231 mμ; $\lambda_{max}^{0.1 N NaOH}$ 261 mμ (12.4 × 10³), $\lambda_{min}^{0.1 N NaOH}$ 225 mμ. $\lambda_{max}^{0.1 N HCl}$ of IVb was transfered to 251 mμ by treatment with nitrous acid.

(iii) 9-(4'-Hydroxybutyl)-6-aminopurine 4'-monophosphate (Vb).—0.6 g. of IVb was dissolved in 3.5 g. of 85 % phosphoric acid and 2.6 g. of phosphorous pentoxide. The mixture was incubated at 60° for 8 hr. To hydrolyze polyphosphates, the mixture was heated for 20 min. at 100° after addition of 30 ml. of water. The whole was adjusted to pH 6.5 with hot, saturated barium hydroxide and barium phosphate separated was filtered hot and washed with 200 ml. of hot water. The filtrate and the washings were combined and evaporated to ca. 10 ml. On readjustment of pH to 7.1 by barium hydroxide, a precipitate appeared, which showed one spot, R_f 0.64 (solvent A') and R_f 0.30 (solvent H'); yield estimated spectrophotometrically, 1.44 mmoles. From the mother liquor, 150 mg. of the precipitate was obtained by addition of 2 volumes of ethanol; total yield 63 %.

(iv) 9-(4'-Hydroxybutyl)-6-aminopurine 4'-triphosphate (VIb).—1.44 mmoles of Vb, 1.78 g. (14.4 mmoles) of 85 % phosphoric acid, 5 ml. of tri-n-butylamine, 7 g. of DCC and 30 ml. of pyridine was caused to react at room temperature for 48 hr. 7.9 g. of urea was recovered. After ether-extraction and evaporation of pyridine, the whole was adjusted to 100 ml. and adsorbed on 12 g. of charcoal. (TOD$_{260}$ 10500). Ion-exchange chromatography: monophosphate, TOD 420, 4.2%; diphosphate, TOD 2090, 20.9%; triphosphate, TOD 7300, 73.0 %; higher phosphates, TOD 190, 1.9%. The triphosphate was obtained as a lithium salt (231 mg.); yield calculated from monophosphate as heptahydrate was 27 %.

Anal. Calcd. for C$_9$H$_{13}$O$_{10}$N$_5$Li$_4$P$_3$·7H$_2$O: total P, 15.6; labile P, 10.4. Found: total P, 15.5; labile P, 10.4. Purity on a weight basis as estimated spectrophotometrically 73 % (ϵ of purine = 14.7 × 10³), estimated from P analysis 79 %. The ratio of base:total P = 1.0:3.1:2.1 (theoretical, 1:3:2). Paper chromatography: R_f 0.26 (solvent A'), 0.59 (solvent H)

6-Methylamino-9-β-D-ribofuranosylpurine 5'-Triphosphate (VIIa).—6-Methylamino-9-β-D-ribofuranosylpurine 5'-phosphate (VIIa) was obtained by the procedure described elsewhere.[17] 400 mg. of Ba-salt of VIIa was freed from Ba by addition of 1 N sulfuric acid in the presence of Na-rhodizonate as an indicator, the supernatant was evaporated with tri-n-butylamine and was caused to react with 1.16 g. of orthophosphoric acid (85%) in 20 ml. of pyridine and 5 ml. of tri-n-butylamine in the presence of 10.4 g. of DCC. Treating of this reaction mixture as described above gave a solution, which had TOD$_{280}$ 3800. After adsorption on 10 g. of activated charcoal,[18] it was washed, eluted with 50 % EtOH–water containing 2 % ammonia (recovery at this stage was 87.3 %), concentrated to a small volume and subjected to ion-exchange chromatography (Amberlite IRA 400, Cl⁻-form, 1.8 × 7 cm.). Elutions with 0.003 N HCl, 0.003 N HCl + 0.05 M LiCl, 0.003 N HCl + 0.075 M LiCl and 0.003 N HCl + 0.15 M LiCl gave, respectively, 2.4 % monophosphate (TOD$_{285}$ 67), 32.9 % diphosphate (TOD$_{265}$ 902), 49.7 % triphosphate (TOD$_{285}$ 1365) and 14.9 % higher phosphates (TOD$_{265}$ 411). The triphosphate fraction was neutralized by 1 M LiOH, evaporated to 5 ml., dissolved in 10 ml. of MeOH and 200 ml. of acetone and stored in a refrigerator overnight. The precipitate was collected, washed with anhydrous EtOH and dry ether and dried on P$_2$O$_5$; yield, 31 mg. Purity calculated on a weight basis as esti-

mated spectrophotometrically (ϵ_{265} of methylaminopurine riboside = 15.9 × 10^3),[48] 62.6 %. Estimated from phosphate analysis (Calcd. for $C_{11}H_{14}N_5O_{13}P_3Li_4\cdot4H_2O$, 12.97. Found:8.02), purity was 62.0 %. Paper chromatography: R_f 0.57 (solvent A), 0.65 (solvent H). Since the main contaminant was LiCl which had no significant effect on the enzymic properties of actomyosin, no further purification was attempted.

6-Dimethylamino-9-β-D-ribofuranosylpurine 5'-Triphosphate (VIIIb).—143 mg. of ribosyldimethylaminopurine monophosphate,[17,49] 0.33 g. of orthophosphoric acid (85 %) and 2.9 g. of DCC was caused to react in 6 ml. of pyridine and 1.5 ml. of tri-*n*-butylamine. Recovery from charcoal was 82.3 %. Ion-exchange chromatography showed the following results. 0.003 N HCl gave 11.5 % monophosphate (TOD$_{268}$ 100), 0.003 N HCl + 0.05 M LiCl gave 17.6 % diphosphate (TOD$_{288}$ 153) and 0.003 N HCl + 0.15 M LiCl gave 71.0 % triphosphate (TOD$_{268}$ 619). The triphosphate fraction was lyophilized and washed with anhydrous MeOH. The residual white powder weighed 22 mg. Purity calculated on a weight basis ($C_{12}H_{18}N_5O_{13}P_3Li_2$), as estimated spectrophotometrically (ϵ_{268} of ribosyldimethylamino-purine = 18.3 × 10^3),[50] 44.8 %. Paper chromatography: R_f 0.15 (solvent A). As the main contaminant was LiCl, no further purification was attempted.

Adenosine 5'-Sulfatopyrophosphate (IX).—200 mg. of ADPNa.4H$_2$O, which was purchased from Sigma Co., was dissolved in 12 ml. of aqueous solution containing 1.2 g. of sodium bicarbonate. It caused to react with 800 mg. of pyridine sulfurtrioxide[51] at 45° for 40 min.[18] Evolution of CO$_2$ along with liberation of pyridine was observed. The whole was poured into 500 ml. of ice water, adjusted to pH 6 with 1 N formic acid and adsorbed on a column (5 × 7 cm.) of charcoal-celite (4:3), at a flow-rate of 4 ml. per min. The column was washed with water (500 ml.) thoroughly until

no turbidity was observed with BaCl$_2$. Elution was achieved by 500 ml. of 50 % EtOH–water solution containing 1 % ammonia. The effluent was adsorbed on a column of Dowex 1 × 2 resin (4 × 7 cm., Cl$^-$- form, 200–400 mesh), washed with 2 volumes of water and eluted with 3 l. of water along with 2 l. of 2 N LiCl by a concave gradient elution technique. After the first peak of ADP (TOD$_{260}$ 2659), the peak of adenosine 5'-sulfatopyrophosphate (TOD 350) was observed. The second fraction was adsorbed on a charcoal-celite (4:3) column (2 × 5 cm.), washed until no Cl$^-$ was detected by aqueous AgNO$_3$ and finally eluted with 300 ml. of 50 % EtOH–water containing 1 % of ammonia. The effluents were concentrated to a small volume in a rotary evaporator at less than 25° and converted to the Li-salt by passing through a column (1 × 1 cm.) of Amberlite IR 120 resin. The effluent and the washings were combined, evaporated to a small volume and lyophilized. 52 mg. of the Li-salt was obtained. Purity estimated spectrophotometrically on a weight basis ($C_{10}H_{12}N_5O_{13}P_2S_1Li_3$), 15.6 %[52]; yield, 1.5 %. The structure of this material was tested by acidic hydrolysis. When it was hydrolyzed with 0.1 N HCl at 37° for 18 min.,[53] ADP (R_f 0.19, solvent S') and SO$_4$ (R_f 0.28, BaCl$_2$-rhodizonate)[54] were detected on the paper chromatogram, accompanied with spots of base and of organic phosphate[55] (R_f 0.57). Paper chromatography:

Compound	Solvent S'	Solvent H'
AMP	0.21	0.27
ADP	.19	.40
IX	.34	.37
ATP	.15	.45
Inorganic sulfate	.28	
Inorganic phosphate	.22	

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(52) Low purity of this material was due to the contamination with LiCl, which were difficult to remove by the reprecipitation technique.

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The Effects of Charged Groups on the Chromophores of Lysozyme and of Amino Acids[1,2,3]

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At acid pH, the difference spectrum of lysozyme, obtained when a solution at one pH is measured against another at a different pH, appears to be due to the effect of the charges of ionizable carboxyl groups on nearby indole chromophores of the molecule. The dependence of the difference spectrum on pH, temperature and ionic strength is presented. A comparison of the difference spectra of lysozyme with those of amino acids containing chromophores found in proteins indicates that, although the effect of the charged groups upon the chromophores appears to occur mainly as an inductive effect through covalent bonds in amino acids, a large part of the charge effect takes place through the solution in the case of lysozyme.

Introduction

The general red shift of the spectra of chromophores present in protein molecules, as compared with the spectra of these same chromophores in smaller molecules, e.g., amino acids, has been known for some time.[4] Some part of the observed modifications of the spectra of a protein's chromophores

must be attributed to the influence of non-charged groups (including the amide bonds) in the protein, while another part must be caused by the presence of charged functional groups on the protein. The effects of charges on the spectra of a protein's chromophores can be observed when ionizable groups of the protein gain or lose protons as the pH of the solution containing the protein is changed.

Apparently almost any change in the environment of a chromophore will produce some change in its spectrum. A discussion of spectral perturbations with particular reference to proteins has been presented.[5] The effect of the solute on the chromo-