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Adenosine 5′-Phosphorothioate. A Nucleotide Analog That Is a Substrate, Competitive Inhibitor, or Regulator of Some Enzymes That Interact with Adenosine 5′-Phosphate*

A. W. Murray and M. R. Atkinson

ABSTRACT: Adenosine 5′-phosphorothioate, an analog of adenosine 5′-phosphate, has been prepared from adenosine and thiophosphoryl chloride in triethyl phosphate in 65% yield and isolated as the sodium salt. The adenosine 5′-phosphate content of this product (measured with adenylate kinase) was less than 0.5% and the content of adenosine derivatives other than the 5′-phosphorothioate (measured as unchanged material after treatment with adenylate deaminase or with 5′-nucleotidase) was 1–3%. Adenosine 5′-phosphorothioate (0.34 mM) was phosphorylated by adenosine triphosphate in the presence of muscle adenylate kinase at less than 0.3% of the rate with adenosine 5′-phosphate at the same concentration, but 0.25 mM adenosine 5′-phosphorothioate increased the adenosine 5′-phosphate concentration for half-maximal activity, $[S]_{0.5}$, from 0.15 to 0.18 mM and decreased the maximal velocity (*V*, *micromoles per minute per milligram of* protein) from 120 to 91 without changing the slope (1.35) of the Hill plot. Adenosine 5′-phosphorothioate was deaminated by adenylate deaminase from rat skeletal muscle. The Michaelis constant, K_m, was 1.6 mM and *V* was 125, 9% of the maximal velocity with adenosine 5′-phosphate ($V = 1330$; $K_m = 0.9$ mM). With 5′-nucleotidase from *Crotalus* venom liberation of adenosine from adenosine 5′-phosphorothioate was much slower than that from adenosine 5′-phosphate ($V = 0.026$ and 1.39, respectively) but K_m for adenosine 5′-phosphorothioate (0.02 mM) was less than that for adenosine 5′-phosphate (0.03–0.035 mM). Adenosine 5′-phosphorothioate is a competitive inhibitor of dephosphorylation of adenosine 5′-phosphate, with inhibitor constant, K_i, of 0.02 mM. Adenosine 5′-phosphorothioate activates the allosteric enzyme yeast diphosphopyridine nucleotide–isocitrate dehydrogenase almost as effectively as the known activator adenosine 5′-phosphate. At 0.31 mM D-isocitrate, half-maximal activation was given by 0.23 mM adenosine 5′-phosphorothioate and 0.19 mM adenosine 5′-phosphate; 0.35 mM adenosine 5′-phosphorothioate decreased $[S]_{0.5}$ for isocitrate from 1.64 to 0.29 mM with little effect on the slope of the Hill plot (3.8–4.0) or on the maximal velocity. Adenosine 5′-phosphorothioate was less effective than adenosine 5′-phosphate as an inhibitor of fructose 1,6-diphosphatase from rat liver. At 0.1 mM fructose 1,6-diphosphate with 10 mM Mg^{2+} 50% inhibition was given by 0.16 mM adenosine 5′-phosphate and 1.6 mM adenosine 5′-phosphorothioate.

Adenosine 5′phosphorothioate was more effective than adenosine 5′-phosphate as an activator of phosphorylase *b* from rabbit skeletal muscle with half-maximal activation of phosphate liberation from 20 mM glucose 1-phosphate at 13 μM adenosine 5′-phosphorothioate and 40 μM adenosine 5′-phosphate. The maximal activation with adenosine 5′-phosphorothioate was 1.3 times that with adenosine 5′-phosphate.

Nucleoside 5′-monophosphates and nucleoside 5′-triphosphates, particularly AMP and ATP, are some of the most important regulators of metabolic processes (for reviews, see Krebs (1964) and Atkinson (1966)). These regulators act by modifying the catalytic activity of regulatory enzymes, and in general a low value of the ATP charge (*i.e.*, of the ratio ([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP]); *cf.* Atkinson and Walton, 1967; Atkinson and Fall, 1967) favors reaction sequences that generate ATP, while a high ATP charge favors reaction sequences that consume ATP. Nucleoside monophosphates and nucleoside triphosphates are readily interconverted in multienzyme systems that contain the corresponding kinases, and there is a need, in

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studies of artificial regulation of metabolic processes, for nucleotide analogs that will mimic the action of natural regulatory nucleotides, but that will be resistant to modification by reactions such as transphosphorylation in the presence of kinases.

Phosphonate derivatives in which the $-PO_2^-$–O–PO_2^-– groups of nucleoside triphosphates are replaced by the stable $-PO_2^-$–CH_2–PO_2^-– group are satisfactory analogs of GTP (Hershey and Monro, 1966) and of ATP (Atkinson and Murray, 1967; Duée and Vignais, 1968), but corresponding analogs of AMP, combining metabolic stability with regulatory activity, have not been described. Myers (1966) has prepared an analog of uridine 5′-phosphate in which the $-CH_2$–O–PO_2^-– group of the nucleotide is replaced by a $-CH_2$–CH_2–PO_2^-– group, and the corresponding derivative of AMP would be of interest, but phosphonates of this type have the disadvantage that their weaker acidity (Myers, 1966) would result in a smaller proportion of the analog being in the same ionic form as a nucleotide at physiological pH. Eckstein (1966) has recently described the preparation of uridine and thymidine 5′-phosphorothioates, which have $-CH_2$–O–PSO_2^{2-} groups in place of the $-CH_2$–O–PO_3^{2-} groups of uridine 5′-phosphate and thymidine 5′-phosphate. The greater acidity of these phosphorothioates (pK_{a_2} = 4.6–4.8) and their resistance to enzymic hydrolysis (Eckstein and Sternbach, 1967) suggested the possibility that AMPS[1] would combine relative metabolic stability with steric and charge properties very similar to those of AMP. AMPS has now been prepared by treatment of adenosine, in triethyl phosphate, with thiophosphoryl chloride. The new nucleotide analog has a high affinity for a number of enzymes that bind AMP, either as a substrate or as a regulator.

Materials and Methods

Preparation of Adenosine 5′-Phosphorothioate. Adenosine (534 mg, 2 mmoles) was suspended in triethyl phosphate (5 ml; British Drug Houses Ltd., Laboratory Reagent) at 100°; the solution was cooled rapidly to 0°, mixed with 0.6 ml of thiophosphoryl chloride (5.8 mmoles; Koch-Light Ltd.), and after 12 hr at 0–2° the resultant suspension was mixed with 20 ml of 10% aqueous barium acetate and kept at 20° for 45 min. Electrophoretic analysis in 0.05 M citrate (Tris, pH 4.8) at this stage indicated the presence of 0.64 mmole of adenosine, 1.36 mmoles of material migrating with 26% greater anionic mobility than AMP (97% pure AMPS, as measured with adenylate deaminase; over-all yield of AMPS, 65%), and about 6 μmoles of material (probably diphosphorothioates) migrating with 80% greater anionic mobility than AMP. The procedure is based on the direct phosphorylation of unprotected nucleosides with $POCl_3$ in triethyl phosphate which Yoshikawa *et al.* (1967) have used to prepare nucleoside 5′-phosphates in yields of about 90% with only traces of 2′- or 3′-phosphates. The solution was brought from pH 1.1 to 9 with 5 ml of triethylamine and the precipitate obtained on addition of 60 ml of 95% ethanol was washed with 70% ethanol (three 40-ml portions) and extracted with water (three 50-ml portions). The aqueous extract was passed through a column (25 × 200 mm) of DEAE-cellulose (HCO_3^- form) and a linear gradient of 250 ml of 0.4 M NH_4HCO_3 into 250 ml of water was passed through the column. Material eluting between 0.08 and 0.16 M NH_4HCO_3 (12,200 ODU at 258 mμ) had λ_{max} 258 mμ; peak fractions were combined, evaporated at 45° (25 mm), and the residue was again dried by evaporation with 50 ml of ethanol, twice with 1 ml of triethylamine in 60 ml of 80% ethanol, and once with 50 ml of ethanol. A solution of the residue in 7 ml of dry methanol was mixed with 6 ml of 1 M NaI in dry acetone containing 1% mercaptoethanol, and the sodium salt was precipitated with 75 ml of acetone. The residue was washed with acetone (four 30-ml portions) and dried at 55°/25 mm. The sodium adenosine 5′-phosphorothioate obtained (0.3 g) contained S:N: adenosine (measured with adenosine deaminase after treatment with 5′-nucleotidase) in the ratios 1.00: 4.97:0.97; the theoretical ratios for AMPS are 1:5:1. The molecular weight, from the S content, was 444. *Anal.* Calcd for $C_{10}H_{12}N_5O_6PSNa_2 \cdot 2H_2O$: 443. In 0.1 N HCl (pH 1.2) AMPS had λ_{max} 257 mμ (mε 14.8), in 0.015 M acetate (Na^+, pH 4.8) it had λ_{max} 259 mμ (mε 14.8), and in 0.015 M NH_4OH (pH 10.1) it had λ_{max} 259 mμ (mε 15.3). At least 99% of the material absorbing at 258 mμ migrated as a single spot on electrophoresis in 0.05 M citrate (Tris, pH 4.8), M_{AMP} 1.26, and in 0.05 M borate (Na^+, pH 8.5), M_{AMP} 1.05, and on chromatography in 66% (v/v) isobutyric acid adjusted to pH 4.2 with ammonia (R_F 0.41), in ethanol–1 M ammonium acetate (pH 7.5) (7:3, v/v) R_F 0.11), and in propan-1-ol–$(NH_4)_2SO_4$–0.1 M sodium phosphate (pH 6.8) (1:30:100, v/w/v) (R_F 0.23). The content of AMP, measured in a coupled assay with lactate dehydrogenase, pyruvate kinase, and adenylate kinase, was not more than 0.5%, and the content of other adenosine derivatives, resistant to hydrolysis by 5′-nucleotidase or deamination by adenylate deaminase, was not more than 1–3%.

Nucleotides and Other Reagents. These were obtained from the Sigma Chemical Co. or were British Drug Houses Analytical Reagent grade.

Adenylate Deaminase. This was prepared from rat skeletal muscle as described by Smiley *et al.* (1967) for the enzyme from rabbit muscle, but with 7.5 volumes of extraction buffer in the initial homogenate. The deaminase was retained by Whatman cellulose phosphate (P-50) when elution with 1 M KCl was attempted in the conditions described by Smiley *et al.* (1967) but was eluted by 1 mM mercaptoethanol–2 M KCl, adjusted to pH 7 with K_2HPO_4, as a single peak with constant specific activity (*ca.* 1330 μmoles of AMP hydrolyzed/

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: AMPS, adenosine 5′-phosphorothioate; M_{AMP}, electrophoretic or chromatographic mobility with respect to that of AMP; V, maximal velocity at saturating substrate concentration; V_{AMP}, maximal velocity with AMP as substrate; V_{AMPS}, maximal velocity with AMPS as substrate; $[S]_{0.5}$, concentration of substrate for half-maximal rate. IMPS, inosine 5′-phosphorothioate; 3-iso-AMP, 3-β-D-ribofuranosyladenine 5′-phosphate; 7-deaza-AMP, tubercidin 5′-phosphate.

min per mg of protein at saturating AMP level, in the assay conditions described below); the enzyme from rabbit muscle had a maximal specific activity, calculated from the results reported by Smiley *et al.* (1967), of 1380.

Other Purified Enzymes. Adenosine deaminase (type I, from calf intestine), 5′-nucleotidase (type II, from *Crotalus adamanteus* venom), glucose 6-phosphate isomerase (grade III, from yeast), and glucose 6-phosphate dehydrogenase (type X, from yeast) were obtained from Sigma Chemical Co. Lactate dehydrogenase, pyruvate kinase, and muscle adenylate kinase were from C. F. Boehringer and Sons.

DPN–Isocitrate Dehydrogenase. This was partially purified from yeast as described by Atkinson *et al.* (1965). The preparation used catalyzed the reduction of 32 mμmoles of DPN/min per mg of protein under the assay conditions described below.

Fructose 1,6-Diphosphatase. This enzyme was prepared from rat liver as described by Taketa and Pogell (1965) and catalyzed the hydrolysis of 0.58 μmole of fructose 1,6-diphosphate/min per mg of protein (see below).

Phosphorylase b. The enzyme was prepared from rabbit muscle as described by Fischer and Krebs (1962). Material crystallized three times was suspended in 0.04 M 3-phosphoglycerate–0.03 M cysteine (K^+, pH 6.8).

Enzyme Assays. Adenylate deaminase activity was measured with the concentrations of AMP and AMPS indicated by following with a Zeiss PMQ II spectrophotometer the decrease in absorbance at 265 mμ at 30° in a 2-mm cell in a system containing 0.6 ml of 0.5 M KCl–1 mM mercaptoethanol–0.02 M potassium cacodylate (pH 6.5) (Smiley *et al.*, 1967) on addition of 0.113 (for AMP) or 2.86 μg (for AMPS) of deaminase. Identification of the deamination product as inosine 5′-phosphorothioate is described in the Results section. With the purified sodium salt of AMPS any residual adenosine derivative resistant to adenylate deaminase was below the limit of detection by this method (approximately 0.6% of the original AMPS).

Adenylate kinase activity was measured as the maximum rate, established after a short lag period (1–2 min), in a coupled assay system (Adam, 1965) with lactate dehydrogenase (10 μg), pyruvate kinase (10 μg), and adenylate kinase (0.5 μg) in 3.0 ml of a solution containing AMP and AMPS at the concentrations indicated, in 0.15 mM DPNH–0.167 mM phosphoenolpyruvate–0.033 mM ATP–23 mM KCl–6.7 mM $MgSO_4$–67 mM Tris-Cl (pH 7.8). In separate experiments it was shown that the rate of oxidation of DPNH, measured at 30° in an Eppendorf photometer at 334 mμ, was proportional to adenylate kinase concentration at this level.

5′-Nucleotidase activity was measured at 30° by following the decrease in absorbance at 265 mμ in a coupled assay with adenosine deaminase (4 μg), 5′-nucleotidase (40 μg), and AMP or AMPS as indicated, in 3.0 ml of 0.1 M Tris-Cl (pH 8.5). In the conditions described, doubling the concentration of 5′-nucleotidase decreased its apparent specific activity in the coupled system by less than 5%.

Isocitrate dehydrogenase activity was measured by following the increase in absorbance at 340 mμ under the assay conditions described by Atkinson *et al.* (1965).

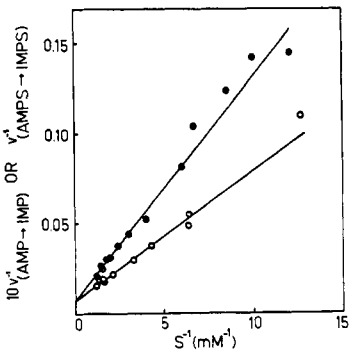

FIGURE 1: Deamination of AMP (○) and AMPS (●) by muscle adenylate deaminase; *v*, micromoles of IMP (○) or IMPS (●) formed per minute per milligram of protein.

Fructose 1,6-diphosphatase was measured in a continuous coupled assay with phosphoglucose isomerase and glucose 6-phosphate dehydrogenase as described by Underwood and Newsholme (1965). The enzyme preparation was diluted with 0.05 M Tris-Cl (pH 8.5)–0.02 M mercaptoethanol immediately before use; assays contained 0.1 mM fructose 1,6-diphosphate and 10 mM $MgSO_4$.

Phosphorylase *b* was assayed at pH 6.8 as described by Cori *et al.* (1955) except that incubations contained 10 μmoles of glucose 1-phosphate in a final assay volume of 0.5 ml.

Results

Deamination of AMPS by Adenylate Deaminase. As reported by Smiley and Suelter (1967) for the enzyme from rabbit muscle, the rate of deamination of AMP is a hyperbolic function of AMP concentration at the high ionic strength used here. The maximal velocity (V_{AMP} = 1330 μmoles/min per mg of protein; Figure 1) and K_m (0.95 mM AMP) are close to the values (V_{AMP} = 1380 μmoles/min per mg of protein; K_m = 0.5–0.7 mM AMP; *cf.* Smiley *et al.*, 1967) reported for the enzyme from rabbit muscle. The deaminase converts AMPS into inosine 5′-phosphorothioate at a lower rate (V_{AMPS} = 125 μmoles/min per mg of protein); the Michaelis constant was 1.6 mM AMPS.

Identification of the Product of Deamination of AMPS as IMPS. On addition of 0.1 ml of adenylate deaminase (0.03 mg) to 2 ml of 22 mM AMPS and 1 ml of 0.5 M KCl–1 mM mercaptoethanol–0.04 M cacodylate (K^+, pH 6.5), deamination, measured from the increase in absorbance at 290 mμ in a 2-mm cell, was complete in 160 min; (at pH 6.5 AMPS and IMPS, like AMP and IMP, have isosbestic points at 223, 251, and 282 mμ; deamination increases the absorbance in the range 283–295 mμ). Barium acetate (2 ml of 10% w/v) and ethanol (15 ml) were added and the pH was brought to 8 with triethylamine. The precipitated barium IMPS was washed with 75% ethanol (three 10-ml portions), dissolved in 20 ml of water, and freed of Ba^{2+} with Chelex-100 resin (Na^+ form; Bio-Rad Laboratories). On electrophoresis in 0.05 M citrate (Tris, pH 4.8) and

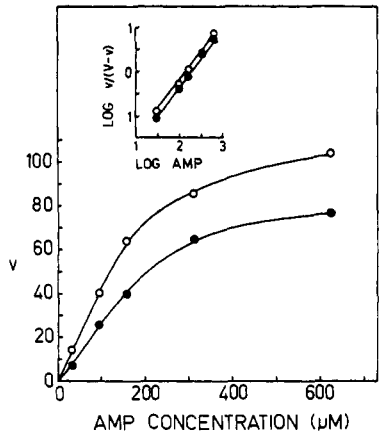

FIGURE 2: Effect of AMPS on the velocity of adenylate kinase. Assays were carried out in the absence of AMPS (○) or in the presence of 0.25 mM AMPS (●).

on chromatography in the ammonium isobutyrate system described above the product migrated as a single ultraviolet-absorbing component with anionic mobility 33% greater than that of IMP and R_F 0.17 (67% of the R_F of IMP). The spectrum was identical with that of IMP at pH 1 and 5. The enzymic deamination product had the same electrophoretic and chromatographic properties as IMPS prepared from inosine and $PSCl_3$ in 45–50% yield by a procedure identical with that described for the preparation of AMPS.

Interaction of AMPS with Adenylate Kinase. At the concentrations of adenylate kinase, pyruvate kinase, and lactate dehydrogenase used in the assay described above adenylate kinase was rate limiting, with the over-all reaction rate proportional to adenylate kinase concentration. As shown in Figure 2, the rate was not a hyperbolic function of AMP concentration, but gave a good fit to a Hill plot with slope 1.35. On addition of

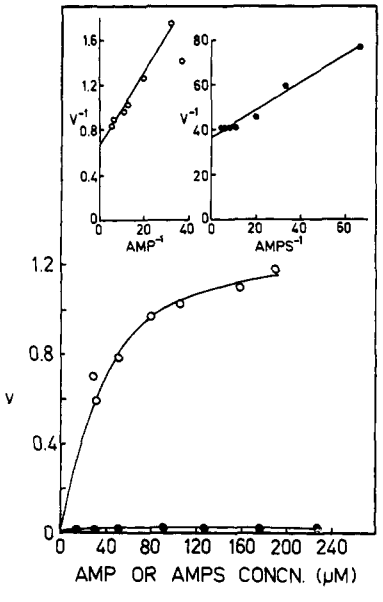

FIGURE 3: Effect of AMP (○) and AMPS (●) concentration on the initial velocity of 5′-nucleotidase; v, micromoles of adenosine formed per minute per milligram of protein.

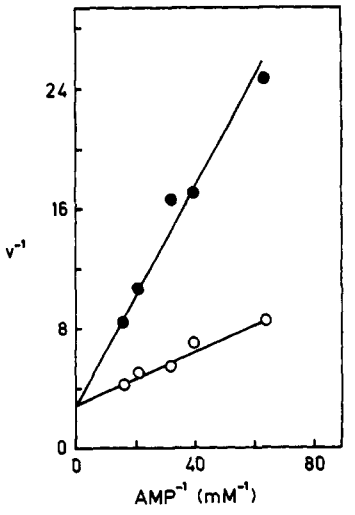

FIGURE 4: Inhibition of 5′-nucleotidase by AMPS. Assays were carried out in the absence of AMPS (○) or in the presence of 51 μM AMPS (●).

AMPS to the coupled assay system there was rapid oxidation of DPNH corresponding to an AMP content of about 0.5% of the AMPS, and this was followed by a very slow oxidation of DPNH. The apparent rate of phosphorylation of 0.34 mM AMPS was less than 0.3% of the rate with AMP at the same concentration. This was at the limit of measurement in this system, and detailed kinetic studies of AMPS as a substrate have not been carried out, but the rate did not interfere with studies of AMPS as an inhibitor of kinase action with AMP as a substrate. AMPS showed no slow irreversible inhibition of adenylate kinase. The rate on addition of 0.15 mM AMP after the complete assay system had been in contact with 0.34 mM AMPS for 100 min was 99% of the rate when AMPS and AMP were added together. As shown in Figure 2, AMPS inhibits adenylate kinase without changing the slope of the Hill plot. AMPS (0.25 mM) decreased V_{AMP} from 120 to 91 μmoles per min per mg of protein and increased $[S]_{0.5}$ from 0.15 to 0.18 mM AMP.

AMPS as a Substrate and Inhibitor of 5′-Nucleotidase. With the coupled assay system described in the Materials and Methods section the over-all rate of hydrolysis and deamination of AMP to inosine was proportional to 5′-nucleotidase concentration within 5%, and the rate of hydrolysis of AMP (Figure 3) was a hyperbolic function of AMP concentration (V_{AMP} = 1.39 μmoles/min per mg of protein; K_m = 0.035 mM). Liberation of adenosine from AMPS was much slower (Figure 3) with V_{AMPS} = 0.026 μmole/min per mg of protein (1.9% of V_{AMP}), but the Michaelis constant was low (K_m = 0.02 mM AMPS). In a separate experiment (Figure 4) 0.05 mM AMPS was a competitive inhibitor of hydrolysis of AMP by the nucleotidase, and the observed values of K_m (0.03 mM AMP) and K_i (0.02 mM AMPS) are in good agreement with the results in Figure 3.

In separate experiments to identify the products formed from AMPS with 5′-nucleotidase it was found that some orthophosphate (7–10% of the adenosine

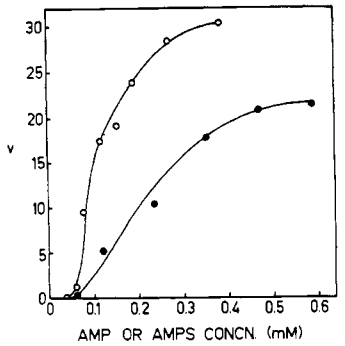

FIGURE 5: Activation of yeast isocitrate dehydrogenase by AMP (○) and AMPS (●); v, millimicromoles of DPN reduced per minute per milligram of protein.

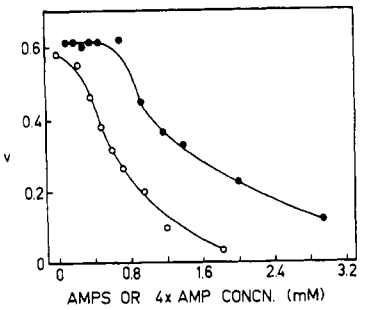

FIGURE 7: The effect of AMP (○) and AMPS (●) on the activity of rat liver fructose 1,6-diphosphatase; v, micromoles of fructose 6-phosphate formed per minute per milligram of protein.

formed, on a molar basis) was freed. The orthophosphate was measured after extraction into isobutyl alcohol as phosphomolybdate (Weil-Malherbe and Green, 1951). Phosphorothioate was detected from the increase in absorbance at 241 mμ in samples diluted in 0.1 N NaOH (Neumann *et al.*, 1965). The low yield of orthophosphate indicates that AMPS is not converted into AMP prior to its hydrolysis by 5′-nucleotidase.

AMPS as an Activator of DPN–Isocitrate Dehydrogenase. In the presence of 0.308 mM isocitrate there was no detectable reduction by DPN by the yeast isocitrate dehydrogenase, but activity was observed when increasing concentrations of AMP or AMPS were added to assay mixtures (see Figure 5). The concentrations of AMP and AMPS to give half-maximal rates were 0.19 and 0.23 mM, respectively; the maximum velocities were determined by reciprocal plots of rate against nucleotide concentration as described by Atkinson *et al.* (1965). The maximum velocity obtained with AMPS (24.7 mμmoles of DPN reduced/min per mg of protein) was 50% of that obtained with AMP (49.5 mμmoles of DPN reduced/min per mg of protein). Hill plots in the presence of either AMP or AMPS had slopes of 1.6 and 2.1, respectively; Atkinson *et al.* (1965) reported a slope of between 1.7 and 2.0 under these conditions. A separate experiment was also carried out in which the concentration of isocitrate was varied in the absence of added activator, in the presence of 0.36 mM AMP, or in the presence of 0.35 mM AMPS; values of $[S]_{0.5}$ were 1.64, 0.17, and 0.29 mM, respectively. The extrapolated maximum velocity was the same in each case (33 mμmoles of DPN reduced/min per mg of protein) and the slope of the Hill plots varied from 3.8 to 4.0 (see Figure 6; *cf.* slopes of 3.8–3.9 reported by Atkinson *et al.*, 1965).

AMPS as an Inhibitor of Fructose 1,6-Diphosphatase. Assays were carried out as described in the Materials and Methods section in the presence of 0.1 mM fructose 1,6-diphosphate and 10 mM $MgSO_4$ at 30°. Control assays carried out without fructose 1,6-diphosphate showed no reaction, indicating no detectable formation of glucose 6-phosphate from glycogen (Krebs and Woodford, 1965). Under these conditions both AMP and AMPS inhibited fructose 1,6-diphosphatase (Figure 7); the concentrations of nucleotide required to give 50% inhibition were 165 μM and 1.6 mM, respectively. At the highest concentrations tested neither AMP nor AMPS inhibited the reduction of TPN when fructose 1,6-diphosphate was replaced by 0.1 mM fructose 6-phosphate in the assay system, indicating that both compounds were inhibiting the activity of fructose 1,6-diphosphatase.

Activation of Phosphorylase b by AMPS. Under standard assay conditions (see Materials and Methods) both AMP and AMPS stimulated the release of P_i from glucose 1-phosphate in the presence of phosphorylase *b* and glycogen (Figure 8). The maximum rates of reac-

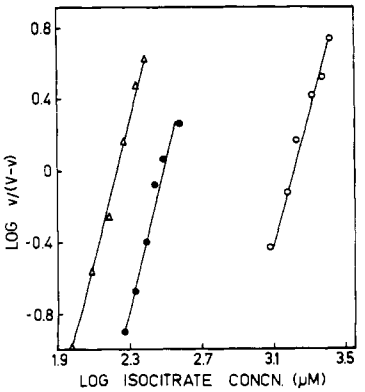

FIGURE 6: Plots of log $(v/(V - v))$ against log isocitrate concentration in the absence of activator (○) or in the presence of 0.36 mM AMP (△) or 0.35 mM AMPS (●).

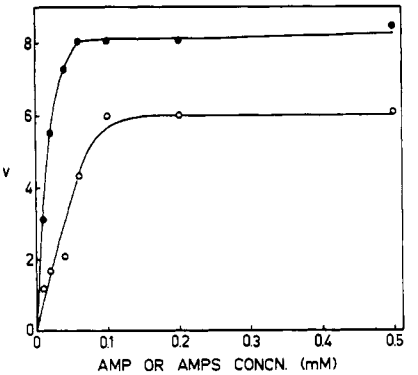

FIGURE 8: Activation of muscle phosphorylase *b* by AMP (○) and AMPS (●); v, micromoles of P_i liberated per minute per milligram of protein.

tion were 6.1 and 8.1 μmoles of P_i released per min per mg of protein for AMP and AMPS, respectively; half-maximal rates were obtained with 40 μM AMP and 13 μM AMPS.

Discussion

Although many nucleotide analogs have been found to act as substrates and competitive inhibitors of enzymes that metabolize nucleotides, there have been few reports of nucleotide analogs that mimic natural regulatory nucleotides by activating or inhibiting regulatory enzymes. 6-Thioinosine 5′-phosphate, 6-thioguanosine 5′-phosphate, and 8-azaguanosine 5′-phosphate inhibit phosphoribosyl pyrophosphate amidotransferase from pigeon liver (McCollister *et al.*, 1964) and it has been proposed that these nucleotides are acting as analogs of the natural regulator GMP (Caskey *et al.*, 1964). It has recently been reported that an extensive series of nucleotide analogs resembles AMP in activating threonine deaminase from *Escherichia coli*, but only formycin 5′-phosphate, 7-deaza-AMP, and iso-AMP had activation constants similar to that of AMP (Nakazawa *et al.*, 1967). These three AMP analogs are substrates of adenylate kinase or related kinases (Umezawa *et al.*, 1967; Acs *et al.*, 1964; Leonard and Laursen, 1965), and the main aim of the present study was to find an analog of AMP that would mimic its regulatory activity, but that would not be phosphorylated by ATP in the presence of adenylate kinase, which is often present in unfractionated systems used for studies of regulatory effects. Mott and Bieber (1968) found that of a number of AMP analogs only 3-iso-AMP and, to a much smaller extent, adenosine 5′-phosphoramidate resembled AMP as activators of phosphorylase *b* (on addition of salmine the structural requirement was much less specific and even GMP was an activator).

The phosphorothioate analog of AMP described here has some advantages over these compounds since it is relatively resistant to the action of adenylate kinase and 5′-nucleotidase. It has not been tested with mammalian hydrolases, but the 5′-phosphorothioate derivatives of uridine and thymidine are resistant to *E. coli* phosphatase and a phosphatase from hog spleen (Eckstein and Sternbach, 1967).

Deamination of AMPS by adenylate deaminase might interfere with its use in some systems, but it is likely that suitable substitution in the purine ring would prevent deamination. In a separate investigation (M. R. Atkinson, A. W. Murray, and M. H. Maguire, unpublished results) it was found that 9-β-D-ribofuranosyl-6-chloropurine 5′-phosphate was converted into IMP by muscle adenylate deaminase ($V/V_{AMP} = 0.035$) but that 2-chloro-AMP was not a substrate. It is likely that the 2-chloro derivative of AMPS would resist both deamination and hydrolysis and might be a useful regulator where deamination is a problem.

The isomeric purity and high yields of 5′-phosphates obtained from $POCl_3$ and ribonucleosides with trialkyl phosphates as solvent (Yoshikawa *et al.*, 1967) are in marked contrast to previous results with this phosphorylating agent, and this effect of the solvent is at present unexplained. Although the yields of phosphorothioates obtained with $PSCl_3$ are lower than those reported for phosphates, the method is convenient and has been applied successfully to a number of ribosides including kinetin riboside and 6-methylthioinosine (A. W. Murray and M. R. Atkinson, unpublished results).

[^{14}C]AMPS, identical in electrophoretic and chromatographic behavior with the material described here, was obtained when ribose was treated in $PSCl_3$ in the standard conditions and the monophosphorothioate fraction was treated successively with ATP plus phosphoribosyl pyrophosphate synthetase and then with [^{14}C]adenine plus adenine phosphoribosyltransferase from Ehrlich cells (A W. Murray and P. C. L. Wong, unpublished results). Eckstein (1966) used triimidazolyl phosphinsulfide as the thiophosphorylating reagent with protected uridine and thymidine derivatives and where subsequent conversion into nucleoside diphosphate and triphosphate analogs is to be carried out (*cf.* Eckstein and Gindl, 1967); that is at present the preferred method.

In previous studies of muscle adenylate kinase, Noda (1958) found a hyperbolic relationship of rate of AMP phosphorylation to AMP concentration, with 8 mM ATP and 8 mM Mg^{2+} at pH 8; the Michaelis constant was 0.26 mM AMP. In the work described here the ATP concentration was 10% of the K_m reported by Noda (1958) and the observed rate is close to that calculated from the earlier work for these substrate concentrations. Kuby *et al.* (1962) calculate from ultracentrifuge studies a maximal binding of 2.3 moles of AMP/mole of muscle adenylate kinase, but it is not known if the nonhyperbolic kinetics reported here (Hill plot slope, 1.35; Figure 2) result from this multiple binding or are a feature of the coupled assay system used in this study.

In this work the well-known activations of yeast DPN–isocitrate dehydrogenase (Hathaway and Atkinson, 1963; Atkinson *et al.*, 1965) and of phosphorylase *b* (for references, see Neufeld and Ginsburg, 1965) and inhibition of fructose 1,6-diphosphatase (for references, see Wood, 1966) by AMP were observed and in each case similar effects were obtained with AMPS. However, although AMPS was a satisfactory analog of AMP with isocitrate dehydrogenase and phosphorylase *b*, it was relatively ineffective as an inhibitor of fructose 1,6-diphosphatase indicating that these enzymes vary in their structural requirements for AMP binding. Thus it will be difficult to obtain a single compound which will be an effective analog in all enzyme systems regulated by AMP. In spite of this limitation AMPS has a favorable combination of stability and affinity for many catalytic or regulatory sites that bind AMP and it may be useful in studies of regulatory mechanisms.

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