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Direct Measurement and Regulation of 3′-Phosphoadenosine 5′-phosphosulfate (PAPS) Generation *in Vitro*

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ABSTRACT. 3′-Phosphoadenosine 5′-phospho[^{35}S]sulfate (PAPS) biosynthesized from inorganic [^{35}S]sulfate and ATP was separated from its radiolabeled precursor by reversed-phase paired-ion HPLC and quantified by on-line radiometric detection. This single-step procedure circumvents several problems inherent in conventional sulfotransferase-coupled assays employed in the measurement of PAPS formation. A good correlation was observed between the rate of PAPS generation assayed in several mammalian tissues measured by direct HPLC-radiometry and by coupling to the sulfation of minoxidil or 4-methylumbelliferone. Both AMP and ADP inhibited the rat liver sulfate-activating enzymes competitively with respect to $MgATP^{2-}$, and the rate of PAPS production was decreased with decreasing ratios of [ATP]:[ADP] and [ATP]:[AMP]. It is possible that these adenine nucleotides regulate sulfate activation by kinetic control and by negative feedback modulation. BIOCHEM PHARMACOL 52;8:1187–1194, 1996.

KEY WORDS. PAPS; direct assay; HPLC-radiometry; regulation

Sulfate conjugation constitutes an important pathway in the biotransformation of xenobiotics and endobiotics with PAPS† serving as the sulfonate group donor [1]. PAPS has also been implicated in the post-translational phosphorylation of proteins [2], thus demonstrating its diversified roles in biological systems. The bioavailability of PAPS had been reported to determine the rate of sulfate conjugation in the gastrointestinal mucosa, avian cornea, human platelets, and mammalian cartilage [3–7]. The measurement of the ability to generate PAPS would be useful in assessing and predicting the feasibility and limitation of sulfate conjugation in various tissues.

The direct procedure of quantification of PAPS by paper chromatography and high voltage paper electrophoresis [8, 9] is time-consuming and impractical for routine analysis. Both the bioluminescent [10] and catalytic PAP-PAPS [11] procedures were indirect measurements and likewise have not been widely employed. The more common approach is to couple the production of PAPS *in vitro* to the sulfation of an acceptor such as harmol, naphthol, 4-MU, *N*-acetyldopamine, or minoxidil [12–16] in a reaction catalysed by ST. This coupling assay is unsatisfactory mainly because ATP, introduced in the initial PAPS-generating step, has a strong inhibitory effect on the subsequent coupled ST reaction [17]. In view of the inherent limitation in these methods, an alternative assay, independent of the coupled ST step, would be preferable. In this paper and in a preliminary report [18], an on-line, paired-ion HPLC-radiometric procedure for quantifying PAPS provides this option. A comparison between the HPLC and coupling assay procedures was made in parallel measurements of PAPS generation in several mammalian tissue extracts. The kinetics and regulatory properties of the sulfate-activating system in rat liver are also examined.

MATERIALS AND METHODS

Chemicals

Sodium [^{35}S]sulfate (539.08, 564.31, and 758.0 mCi/mmol; radiochemical purity of 99.00%) and [^{35}S]PAPS (1.92 and 2.4 Ci/mmol of 96.0 and 99.6% radiochemical purity, respectively) were purchased from the New England Nuclear Corp. (Boston, MA, U.S.A.). DTT, 4-MU, minoxidil, and nucleoside mono-, di-, and triphosphates were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Tetrabutylammonium perchlorate and sodium chlorate were from Fluka AG (Buchs, Switzerland). Methanol of HPLC grade was obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Adenosine 5′-phospho[^{35}S]sulfate ([^{35}S]APS) was

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† *Abbreviations:* PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PAP, 3′-phosphoadenosine 5′-phosphate; ST, sulfotransferase; 4-MU, 4-methylumbelliferone; PP_i, pyrophosphate; DTT, dithiothreitol; PdAPS, 3′-phosphodeoxyadenosine-5′-phosphosulfate; and PUPS, 3′-phosphouridine-5′-phosphosulfate.

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prepared as described earlier [16]. All other chemicals of analytical grade were from the usual commercial sources.

Preparation of Enzyme Extracts

A tissue homogenate (20–40%, w/v) was prepared in cold 0.15 M KCl containing 3 mM DTT from the liver, kidney, or brain of the rat, mouse, guinea pig, and monkey and from the human skin. The supernatant obtained after centrifugation at 108,000 *g* for 30 min was employed as the enzyme source for sulfate activation. Animal and human skin enzyme extracts were prepared as described by Wong *et al.* [19]. The high-speed supernatant of rat liver was used as a source of ST in the assay of PAPS.

Protein Determination

The protein contents of the enzyme preparations were measured by the method of Bradford [20] using the Bio-Rad reagent with bovine serum albumin as standard.

Assay Conditions for PAPS Generation from Inorganic [^{35}S]Sulfate

The reaction mixture contained the following chemicals with their final concentrations in parentheses: 10 μL sodium [^{35}S]sulfate (0.12 mM), 20 μL each of Mg^{2+} (9.3 mM) in DTT (2 mM) and ATP (9.3 mM) in a final volume of 300 μL of 50 mM glycine-NaOH buffer, pH 8.6. The reaction was started with 10–25 μL (containing 100–150 μg protein) of each of the enzyme extracts. Controls were carried out in parallel with the omission of either ATP or Mg^{2+} or by the addition of boiled enzyme extract. At 10 min, the reaction was stopped by boiling for 1 min, and precipitated proteins were pelleted by centrifugation. Aliquots of the supernatant were used for the quantification of [^{35}S]PAPS by HPLC-radiometric analysis or by coupling to the sulfation of 4-MU or minoxidil.

Quantification of [^{35}S]PAPS by Direct HPLC-Radiometry

The HPLC system comprised a Hewlett-Packard 1090 LC model connected to a Flo-one Beta radioactive flow detector (Radiomatic Instruments and Chemical Co. Inc. now part of the Packard Instrument Co., Meriden, CT, U.S.A.) with a 0.25 mL solid cell. The mobile phase, which was modified from that reported by Pennings and van Kempen [21], consisted of 4 mM tetrabutylammonium perchlorate and 30 mM KH_2PO_4 buffer with 20–25% methanol, pH 7.0. The flow rate was maintained at 0.8 mL/min. [^{35}S]PAPS was separated from inorganic [^{35}S]sulfate on a Nova-Pak C_{18} column (4 μm, 3.9 × 150 mm) and quantified by extrapolating from the integrated peak areas compared to those of a standard curve of 3.6 to 36 pmol inorganic [^{35}S]sulfate obtained at the same time.

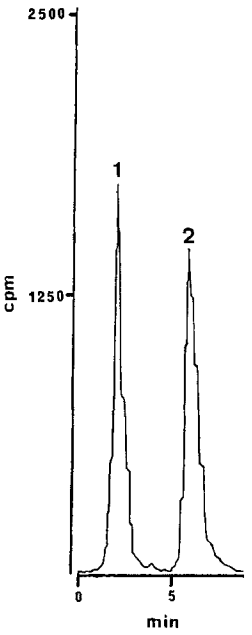

FIG. 1. HPLC chromatogram illustrating the separation of inorganic [^{35}S]sulfate/[^{35}S]APS (peak 1) and [^{35}S]PAPS (peak 2).

Measurements of [^{35}S]PAPS by the ST Reaction

COUPLING TO SULFATION OF 4-MU. To an aliquot of the supernatant from the PAPS-generating step was added 1.4 μM 4-MU. The ST reaction was initiated by the addition of 100 μL of a mixture containing a 1:1:1 ratio, by volume, of rat liver cytosol, 0.2 M EDTA, and 0.2 M PP_i together with 15 μL of 250 mM sodium chlorate. The final volume of the assay mixture was made up to 350 μL with 50 mM

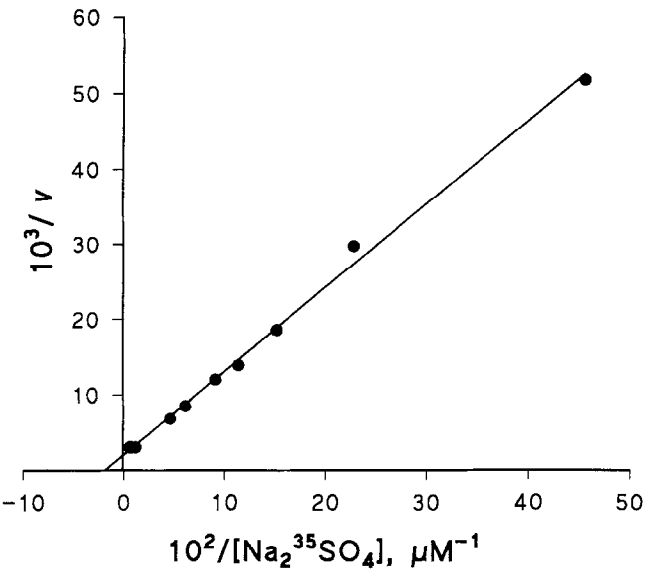

FIG. 2. Lineweaver–Burk plot of PAPS-generating activity in rat liver cytosol where the velocity (*v*) was expressed in pmol [^{35}S]PAPS/min/mg protein against 2.2 to 176 μM sodium [^{35}S]sulfate in the presence of 9.3 mM ATP-$MgCl_2$.

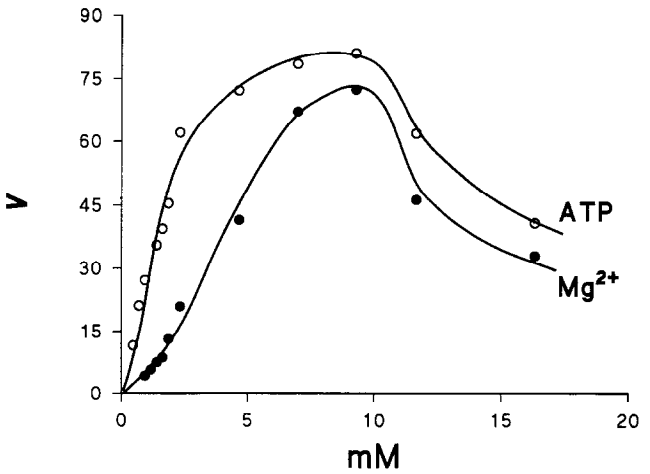

FIG. 3. Effects of Mg^{2+} and ATP concentrations on the velocity (*v*) of PAPS generation expressed in pmol [^{35}S]PAPS/min/mg protein, measured at 9.3 mM ATP and Mg^{2+}, respectively.

phosphate buffer, pH 7.2. Sodium chlorate, at a final concentration of 10 mM, arrested sulfate activation by inhibiting ATP-sulfurylase [22] present in the crude rat liver supernatant. As explained previously [5], EDTA was introduced to chelate Mg^{2+} required for both ATP sulfurylase and APS kinase, while the additional inclusion of PP_i (a product of the ATP sulfurylase reaction) was found to be necessary to *completely* inhibit PAPS generation *in vitro*. The reaction was carried out at 37° for 15 min and terminated by boiling for 1 min. The formation of 4-MU sulfate was determined by measuring the decrease in fluorescence of 4-MU using an HPLC-fluorimetric procedure, described earlier [14]. The mobile phase, composed of methanol:water (3:2, v/v), was delivered at a flow rate of 0.3 mL/min through a microbore column (2.1 × 100 mm) containing Hypersil-ODS of 5 μm particle size. The fluorescence of the eluates was monitored at excitation and emission wavelengths of 319 and 447 nm, respectively. The amount of PAPS produced was extrapolated from a standard curve corresponding to 18–180 pmol PAPS under identical assay conditions.

COUPLING TO SULFATION OF MINOXIDIL. This was carried out by the method of Wong and Wong [16] with slight modification. One hundred microliters of an 8 mM solution of minoxidil was introduced to an aliquot of the supernatant obtained from the PAPS-generating step in a total volume of 380 μL of phosphate buffer (pH 7.4). As in the 4MU–ST assay, the sulfation of minoxidil was carried out by the addition of 100 μL of a mixture of rat liver extract, 0.2 M EDTA, and 0.2 M PP_i in a ratio of 1:1:1 (by volume) together with 20 μL of 250 mM sodium chlorate. At the end of a 10-min incubation, 0.4 mL ammonium hydroxide was introduced to the reaction mixture followed by the extraction of minoxidil sulfate with 1 mL acetate. The radioactivity in 0.5 mL of the organic phase was measured by liquid scintillation counting. The amount of [^{35}S]PAPS produced was quantified as described earlier [5, 16].

Overall Sulfate Conjugation of Other Substrates from Sodium [^{35}]Sulfate and ATP

The transfer of [^{35}S]sulfate from [^{35}S]PAPS generated *in vitro* to other acceptors was studied by the inclusion of other substrates in the reaction assay mixture. The overall sulfation of each of the following substrates: 50 μM dopamine or *N*-acetyldopamine, 2.5 μM *p*-nitrophenol, and 250 μM harmol was carried out in the presence of 0.12 mM sodium [^{35}]sulfate, 9.3 mM each of Mg^{2+} and ATP, 2 mM DTT using a rat liver cytosolic extract containing 130 μg protein per assay incubate. The total volume was made up to 200 μL with 50 mM glycine-NaOH buffer of pH 9.0 for dopamine and *N*-acetyldopamine, pH 8.6 for *p*-nitrophenol, and pH 9.4 for harmol. In the reaction with dopamine as substrate, *trans*-2-phenylcyclopropylamine at a final concentration of 1 mM was preincubated with the rat liver enzyme preparation to inhibit monoamine oxidase activity [23]. After 15 min of incubation at 37°, the reaction was stopped by boiling for 1 min, and the formation of each ^{35}S-labeled sulfate conjugate was detected by HPLC-radiometry as described below.

HPLC-Radioisotopic Detection of [^{35}S]Sulfate Conjugates

For the analysis of the conjugated [^{35}S]sulfates of dopamine, *N*-acetyldopamine, *p*-nitrophenol, and harmol, the mobile solvent systems employed were, respectively, 10 mM NaH_2PO_4 buffer (pH 6.8) with 7, 15, 25% (isocratic system) and with 45% of methanol following an initial step of 20% methanol for 4 min. Each of the solvent systems was delivered at 1 mL/min through a 4.6 × 200 mm column packed with Hypersil-ODS of 5 μm particle size. The radiolabeled conjugates were detected by a Flo-one Beta radioactive flow detector.

TABLE 1. Kinetic constants of rat liver sulfate-activating enzymes with ATP or dATP as the precursor

Substrate	K_m(mM)	V_{max} [nmol · min^{-1} · (mg protein)$^{-1}$]	(V_{max}/K_m) × 10^9 [mL · min^{-1} · (mg protein)$^{-1}$]
ATP-$MgCl_2$	0.50	0.98	1.96
dATP-$MgCl_2$	0.16	0.17	1.06

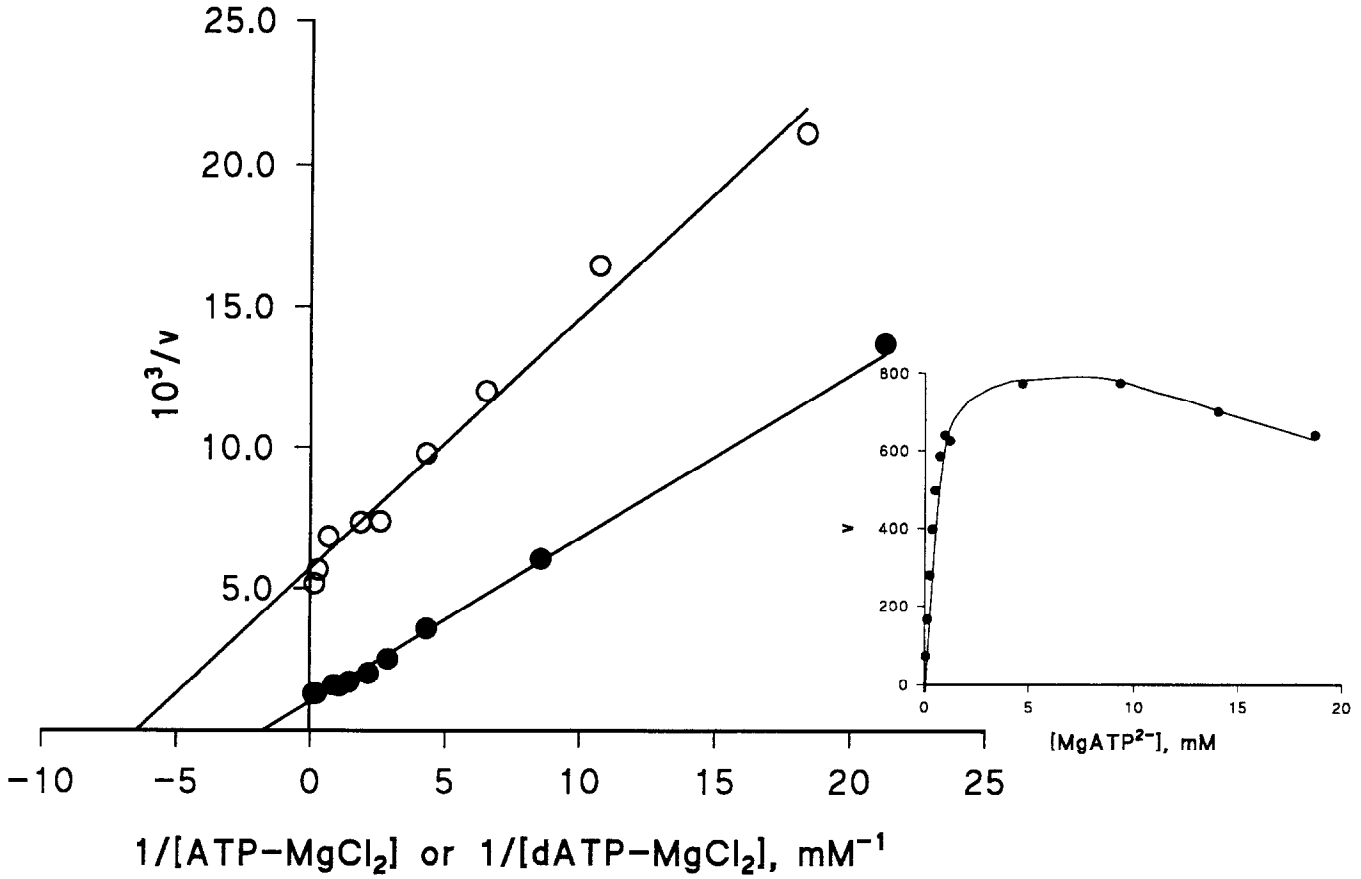

FIG. 4. Lineweaver–Burk plot of PAPS- and PdAPS-generation where *v* is expressed in pmol [^{35}S]PAPS or [^{35}S]PdAPS/min/mg protein measured between 0.047 and 9.3 mM ATP-$MgCl_2$ (●), and between 0.055 and 7.8 mM dATP-$MgCl_2$ (○) at 0.12 mM sodium [^{35}S]sulfate. Data points for concentrations above 9.3 mM ATP-$MgCl_2$ where there was inhibition (inset) were omitted from the Lineweaver–Burk plot.

RESULTS

HPLC-Radiometric Analysis of [^{35}S]PAPS

The incubation of enzyme extracts with ATP and inorganic [^{35}S]sulfate resulted in the formation of a ^{35}S-labeled product when analysed by reversed phase paired-ion HPLC-radiometry. The product was identified as [^{35}S]PAPS based on its co-elution with the authentic ^{35}S-labeled standard and its ability to form sulfate conjugates of the following acceptors: minoxidil, 4-MU, dopamine, *p*-nitrophenol, harmol, and *N*-acetyldopamine. The HPLC chromatographic separation of [^{35}S]PAPS (t_r = 6.0 min) from inorganic [^{35}S]sulfate/[^{35}S]APS (t_r = 2.3 min) is depicted in Fig. 1; [^{35}S]APS co-migrated with inorganic [^{35}S]sulfate under the chromatographic conditions employed.

Kinetic Data of PAPS Generation by Rat Liver

PAPS biosynthesis exhibited an optimum pH of 8.6 when measured in rat liver cytosol by the HPLC-radiometric procedure. There was a linear relationship between PAPS-generating activity and protein concentration up to 130 μg per assay incubate. Under optimal assay conditions, the production of [^{35}S]PAPS progressed linearly for 10 min of incubation. The apparent K_m value for inorganic sulfate determined from the Lineweaver–Burk plot [24] as analysed by the Enzpack program [25] was 53 μM (Fig. 2). Maximum enzyme activity was observed at a 9.3 mM concentration of both ATP and Mg^{2+} (Fig. 3), suggesting that the substrate was an $MgATP^{2-}$ complex. The sigmoidal velocity profiles observed with [Mg^{2+}] or [ATP] (Fig. 3) could be ascribed to the inhibitory effect of uncomplexed (or free) ATP or Mg^{2+}, respectively [26]. The enzyme followed classical Michaelis–Menten kinetics when assayed with equimolar concentrations of ATP and Mg^{2+}, giving an apparent K_m of 0.5 mM for $MgATP^{2-}$ (Table 1, Fig. 4). The degree of inhibition observed above 9.3 mM $MgATP^{2-}$ complex was small (Fig. 4, inset), in contrast to the potent inhibitory effects of free ATP or Mg^{2+} (Fig. 3).

Measurement of PAPS Biosynthesis by Three Independent Assay Procedures

The optimum assay conditions established for the rat liver sulfate-activating system were employed for the measurement of PAPS generation in tissue extracts of liver, kidney, brain, and skin of rat, guinea pig, monkey, and mouse, and of human skin. The radiolabeled "active sulfate" produced was quantified by the direct HPLC-radiometric method and

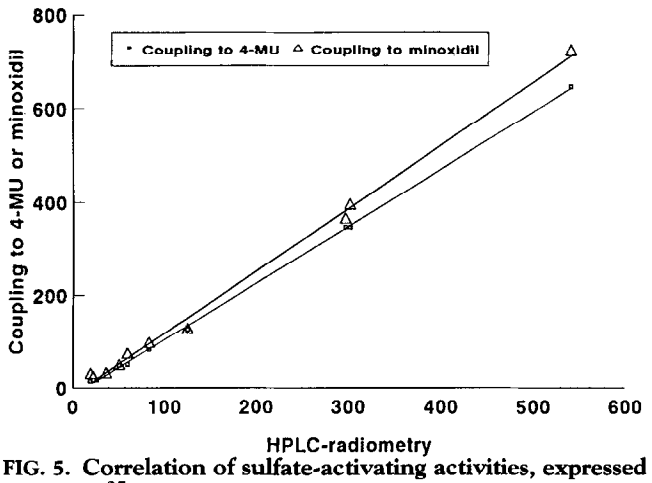

FIG. 5. Correlation of sulfate-activating activities, expressed in pmol [^{35}S]PAPS/min/mg protein, measured in mammalian tissue extracts by HPLC-radiometry and by ST assays coupled to the sulfation of minoxidil and 4-MU.

by transferring the sulfonate group to 4-MU or minoxidil. There was a good correlation between the rate of PAPS generation measured in these tissue extracts by the three independent assay procedures (Fig. 5). For each set, a correlation coefficient of $r = 0.99$ was obtained by the linear regression equation.

Effects of Nucleoside Triphosphates on PAPS Generation in Vitro

When dATP or UTP was used in lieu of ATP, a labeled product, presumably [^{35}S]PdAPS (3′-phosphodeoxyadenosine 5′-phospho^{35}sulfate) or [^{35}S]PUPS (3′-phosphouridine 5′-phospho^{35}sulfate) was formed. Each had a retention time of 5.6 min, migrating faster than authentic [^{35}S]PAPS. The reaction with UTP was not investigated further as its activity was 6-fold lower than that with dATP. The apparent V_{max}/K_m ratios for dATP and ATP were 1.06 and 1.96, respectively (Fig. 4, Table 1). Since the commercially prepared dATP (of 99% chemical purity) would have to contain 55% ATP by weight (calculation based on Equation 14 in appendix to Ref. 22) to produce the above kinetic ratio, the enzyme activity observed with dATP was most unlikely to be contributed to contaminating ATP. To ascertain whether the putative [^{35}S]PdAPS is an "active sulfate," sulfate conjugation of the following prototype substrates was also carried out by employing the [^{35}S]PdAPS biosynthesized *in situ* from dATP-$MgCl_2$ and inorganic [^{35}S]sulfate: 50 μM dopamine (pH 9.4), 50 μM *N*-acetyldopamine (pH 9.0), 2.5 μM *p*-nitrophenol (pH 8.6), and 250 μM harmol (pH 9.0). All the above substrates were labeled with [^{35}S]sulfate on analysis by HPLC-radiometry (Table 2). The rates of the overall sulfate conjugation of dopamine, *N*-acetyldopamine, *p*-nitrophenol, and harmol by dATP with inorganic [^{35}S]sulfate were 13.6, 27.4, 20, and 29.1%, respectively, relative to the reactions carried out with ATP at the same time under identical assay conditions. With ATP, the values of overall sulfate conjugation of dopamine, *N*-acetyldopamine, *p*-nitrophenol, and harmol were, respectively, 230, 344, 54, and 323 pmol sulfate conjugate formed/min/mg protein. The data seemed to suggest that inorganic sulfate was converted by dATP to PdAPS, which then served as a cosubstrate for sulfate conjugation. However, dATP was less efficient than ATP in this bioactivation. As a substrate, ATP could not be replaced by CTP or GTP. In fact, both GTP and CTP inhibited PAPS generation in a concentration-dependent manner (Fig. 6a).

Effects of Nucleoside Mono- and Diphosphates on Sulfate Activation

The inhibition of the sulfate-activating activity in rat liver cytosol by different nucleotide analogues is presented in Fig. 6b. Among the diphosphates examined at 2 mM, 5′-ADP was the most potent inhibitor. This approximates the intracellular hepatic concentration of 1 to 1.4 μmol/g wet weight (or 1.4 to 2 mM) in the rat liver [27]. Compared to that of 5′-ADP, a higher concentration of 3′,5′-ADP, 5′-UDP, and 5′-GDP was required to elicit 50% inhibition. For the AMP derivatives, the naturally occurring analogue 5′-AMP was the most effective inhibitor. Adenosine and PAPS had no effect.

TABLE 2. HPLC separation and identification of [^{35}S]PAPS or [^{35}S]PdAPS and ^{35}S-labeled sulfoconjugates of dopamine, *N*-acetyldopamine, *p*-nitrophenol, and harmol using 10 mM NaH_2PO_4 buffer (pH 6.8) and methanol

	Retention time (min)		
Substrate	[^{35}S]PAPS or [^{35}S]PdAPS	Sulfate conjugate	% Methanol
Dopamine	2.6	5.9	7 (isocratic)
N-Acetyldopamine	2.6	5.4	15 (isocratic)
p-Nitrophenol	2.6	5.6	25 (isocratic)
Harmol	2.6	9.3	45 (step gradient)*

* Refer to text.

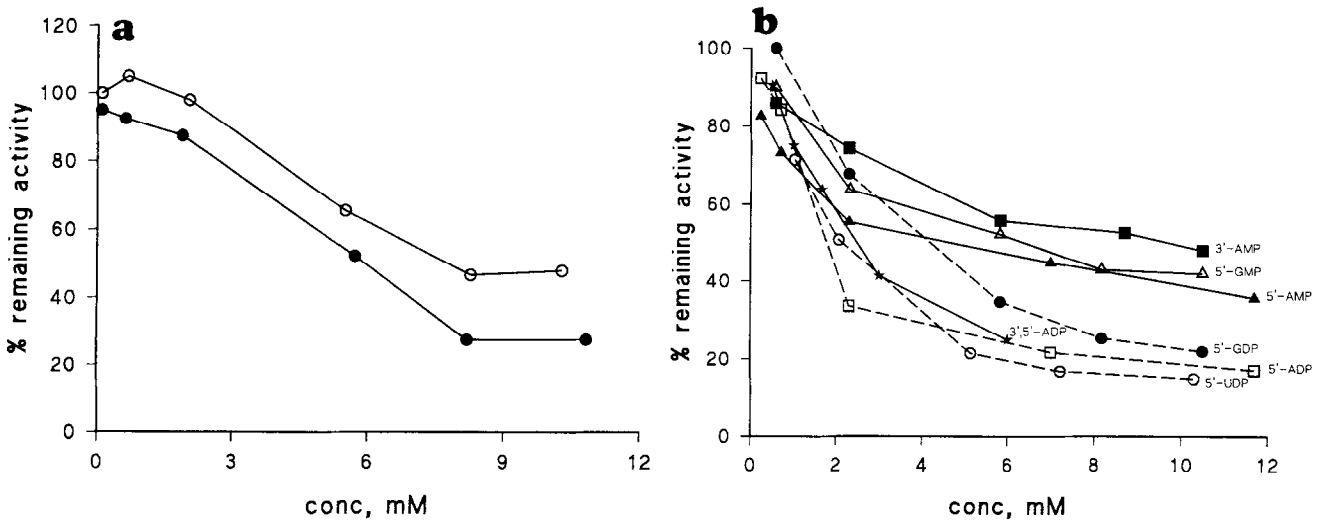

FIG. 6. Effects of (a) CTP (○) and GTP (●) and (b) nucleoside mono- and diphosphates on the PAPS-generating activity of rat liver cytosol. The mono- and diphosphates were: (■) 3′-AMP; (□) 5′-ADP; (▲) 5′-AMP; (△) 5′-GMP; (●) 5′-GDP; (○) 5′-UDP; and (★) 3′,5′-ADP.

The nature of inhibition by 5′-ADP and 5′-AMP was determined from double-reciprocal plots. 5′-ADP was a mixed-type inhibitor with respect to $MgATP^{2-}$ (Fig. 7a), whereas 5′-AMP was competitive with $MgATP^{2-}$ (Fig. 7b). Inhibition of enzyme activity was also observed by decreasing the ratios of [ATP]:[AMP] and [ATP]:[ADP] in the reaction incubates (Fig. 8).

DISCUSSION

The hallmark of the HPLC-radioisotopic method described in this paper is the direct measurement of PAPS, which thus eliminates a number of inherent problems encountered in the ST-coupled assays, such as the inhibitory effect of (a) endogenous inhibitors in tissue extracts [28] employed for sulfate-activation, (b) ATP and its analogues [17], and (c) PAP [17] which increases as the coupled ST reaction progresses. Likewise, the standard solution of PAPS used in the coupled ST assay must also be free from PAP. Attempts to decrease or eliminate the interference by sulfate-activating enzymes present in the crude ST extract employed in the subsequent coupled reaction [5, 14–16] can be dispensed with. The good correlation coefficients of data obtained by HPLC-radiometry and by coupling to the sulfation of 4-MU or minoxidil (Fig. 5) illustrated the reliability of both methods in the quantification of PAPS biosynthesis *in vitro*.

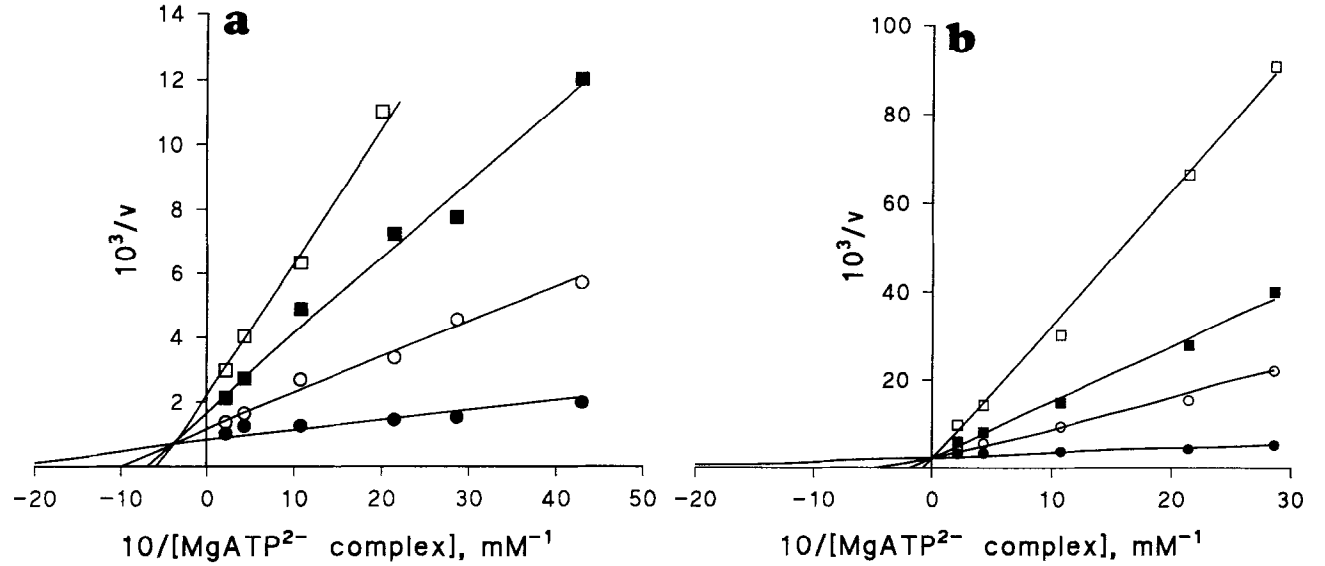

FIG. 7. Inhibition of sulfate activation of rat liver cytosol (expressed in pmol [^{35}S]PAPS/min/mg protein) by (a) ADP and (b) AMP obtained in the range of 0.23 to 4.7 mM $MgATP^{2-}$. Concentrations of ADP employed were 0.7 mM (○), 1.4 mM (■) and 2.3 mM (□), and those of AMP were 0.7 mM (○), 2.3 mM (■) and 9.3 mM (□), as compared with controls without ADP or AMP (●).

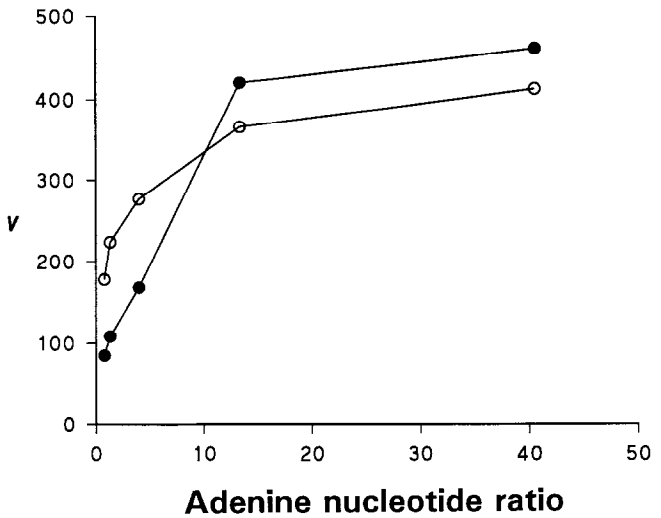

FIG. 8. Effects of a change in the ratios of [ATP]:[ADP] (●) and [ATP]:[AMP] (○) on the velocity *v*, expressed in pmol [^{35}S]PAPS/min/mg protein, of PAPS generation in rat liver cytosol. The concentration of ATP in the assay incubate was 9.3 mM.

Based on the direct and two different coupled-ST assays described, the rate of PAPS generation in rat liver cytosol *in vitro* was 637 pmol [^{35}S]PAPS/min/mg protein, which was comparable to other hepatic values published previously [29]. This value could be translated to approximately 43 nmol [^{35}S]PAPS/min/g liver (based on our calculation of 68 mg cytosolic protein/g wet weight of liver), which was of the same order of magnitude as those of 75–100 nmol/min/g liver predicted *in vivo* and in perfusion studies [30, 31]. The rate of PAPS generation is kinetically modulated by the ratios of [ATP]:[ADP] and [ATP]:[AMP] (Fig. 8). The reported values of [ATP]:[ADP] in rat liver were 1.8 to 2.4 and those of [ATP]:[AMP] were 6 to 9.5 in starved and fed animals [27]. These ratios fall in the range where the rate of PAPS generation could be regulated (see Fig. 8). Thus, control of PAPS biosynthesis by these adenosine nucleotides may be physiologically important. As in most ATP-utilizing reactions, the negative feedback inhibition of ADP and AMP provides a system for ATP conservation which is particularly crucial in sulfate activation which consumes two molecules of ATP per molecule of PAPS biosynthesized. ADP exhibited a mixed-type of inhibition, whereas AMP was competitive with respect to $MgATP^{2-}$ (Fig. 7).

The optimum ratio of $[Mg^{2+}]$ to [ATP] for PAPS formation was 1:1 (Fig. 3), suggesting that the true substrate for PAPS generation in rat liver was $MgATP^{2-}$ complex as was found in fungi [32, 33], yeast [34], and rat skin [16]. There was indirect evidence that ATP-sulfurylase from mouse mastocytoma, spinach leaf, and rat liver could utilize dATP as an alternative substrate [22, 35, 36]. The enzymatic formation by rat liver cytosol of a putative PdAPS that exhibited similar chromatographic properties to PAPS was identified by HPLC-radiometry. Although the affinity for dATP was higher than that for ATP, the V_{max} and V_{max}/K_m values for dATP were lower than those obtained with ATP (Table 1). Thus, from kinetic consideration, the liver has a low capacity and low efficiency to bioactivate sulfate by dATP. This is compounded by the low intracellular concentration of 1 μM of this nucleotide in resting cells [37], which is 100-fold less than its K_m value. On the other hand, the intracellular concentration of ATP of 3–10 mM [37] is not a limiting factor. Nevertheless, PdAPS can be considered as an "active sulfate" based on its ability to sulfoconjugate a number of conventional substrates. Likewise, several analogues of the 3′-phospho- and 2′-phosphoadenosine 5′-phosphosulfate have been reported to serve as cosubstrates for estrogen sulfotransferase [38, 39]. The major structural requirement for these sulfate derivatives to serve as a sulfo-group donor appears to be the 5′-phosphosulfate moiety.

Measured in the combined ATP-sulfurylase and APS kinase reactions, the K_m of 53 μM for sodium sulfate was half the value of 100 μM reported for the single ATP-sulfurylase step [40]. If indeed ATP-sulfurylase and APS kinase in rat liver is a bifunctional protein as demonstrated in rat chondrosarcoma and *Rhizobium meliloti* [41, 42], the physically linked entity could have enhanced affinity for inorganic sulfate. This provides a possible explanation for the observation that tissue PAPS was not affected under resting conditions when hepatic inorganic sulfate was reduced to 360 μM, another reported K_m value for ATP-sulfurylase [43]. Although hydrolysis of GTP appears to drive APS formation in *Escherichia coli* and *R. meliloti* [42, 44], GTP did not exhibit this action in the PAPS-generating system in rat liver cytosol *in vitro* nor in yeast, fungi, and spinach [45, 46].

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