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BIOSYNTHESIS OF 3′-PHOSPHOADENOSINE-5′-PHOSPHOSULFATE (PAPS) IN RAT SKIN

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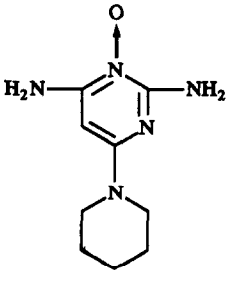
Abstract—A rapid and sensitive radiometric assay was developed for the measurement of 3′-phosphoadenosine-5′-phosphosulfate (PAPS) biosynthesis in rat skin extract. The formation of PAP^{35}S from sodium ^{35}sulfate and ATP was quantified by the transfer of the ^{35}sulfate to minoxidil by rat liver minoxidil sulfotransferase (MST). The assay is sensitive enough for the detection of as little as 2 pmol of PAP^{35}S. The PAPS-generating system showed a pH optimum of 8.6, with an apparent K_m value of 1 mM for the ATP–Mg^{2+} complex and 68 μM for sodium ^{35}sulfate. ATP and Mg^{2+}, present individually or together in equimolar concentrations, were inhibitory above 8 mM. Excess (or free) ATP was a competitive inhibitor with respect to the ATP–Mg^{2+} complex; the apparent K_i measured was 0.32 mM. The specific activity of the PAPS-generating system, measured in rat skin cytosol was 0.15 nmol PAPS/min/mg protein. The importance of PAPS generation in detoxification and bioactivation of xenobiotics in skin is discussed.

Key words: PAPS generation, rat skin, minoxidil sulfotransferase, phenolsulfotransferase

The skin constitutes the largest organ in the body. It possesses an array of detoxicating enzymes whose activities have been reported to be generally low [1] but nevertheless were measurable in reconstituted epidermis [2]. Xenobiotics are known to be metabolized by both Phase I and Phase II reactions in the skin, of which biotransformation by oxidation, reduction, hydrolysis and glucuronidation have been extensively studied [3]. In contrast, reports on sulfate conjugation were limited to the identification of sulfate conjugates of dehydroepiandrosterone (DHA) [4, 5], 7,8-diol of benzopyrene [6], 3-methoxytyramine [7], *p*-nitrophenol, cholesterol [5, 8] and minoxidil [9].



Minoxidil

Minoxidil sulfate is believed to be the active metabolite in promoting hair growth [9] and MST† (activity) has been identified in the hair follicles of the stump-tailed macaque [10], the outer root sheath of rat hair follicles [11] and in human epidermal keratinocytes [12]. Recent studies in our laboratory have demonstrated that rat skin cytosol, besides containing MST activity, has the ability to form minoxidil sulfate when supplemented with ATP and inorganic sulfate [13] suggesting that the skin has the potential of generating PAPS. This communication addresses the activation of sulfate and presents a simple radiometric assay for the measurement of PAPS generation *in vitro* by rat skin cytosol by coupling the "active sulfate" formed to the rat liver MST reaction [14, 15]. In addition, the utilization of PAPS by rat skin PST was also demonstrated with a number of conventional acceptor substrates.

MATERIALS AND METHODS

Chemicals. Sodium ^{35}sulfate (368.05 mCi/mmol) and PAP^{35}S (2.5 Ci/mmol and 1.52 Ci/mmol of 99.2 and 98.3% radiochemical purity, respectively) were purchased from New England Nuclear Corporation (Boston, MA, U.S.A.). Minoxidil, ATP, DTT, dopamine, NADA, *p*-nitrophenol, hexokinase (Type C-300 from bakers yeast, containing 305 U/mg protein; 1 unit will phosphorylate 1 μmol glucose per min at pH 8.5 at 25°) and sulfatase (Limpets, type V) were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Adenosine 5′-phospho^{35}sulfate (AP^{35}S) was prepared by hydrolysing PAP^{35}S with 3′-nucleotidase (Sigma, Type III, from rye grass) [16] or alkaline phosphatase (Sigma, Type I, from calf intestine) [17]. Harmol hydrochloride was from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.)

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† Abbreviations: PAPS, 3′-phosphoadenosine-5′-phosphosulfate; ST, sulfotransferase; PST, phenolsulfotransferase; NADA, *N*-acetyldopamine; MST, minoxidil sulfotransferase; DTT, dithiothreitol; 4-MUS, 4-methylumbelliferyl sulfate.

and tetrabutylammonium perchlorate was purchased from Fluka AG (Buchs, Switzerland). All other chemicals of analytical grade were from the usual commercial sources.

Preparation of enzyme extracts from rat skin. Skin from the back of a Wistar rat was excised and the hair removed with an electric shaver. The subcutaneous fat was discarded and the skin was cut into small pieces. A 40% homogenate was prepared in cold 0.15 M KCl containing 3 mM DTT using the Polytron tissue homogenizer (Kinematica GmbH, Luzern, Switzerland). A further step of homogenization with a motorized tight-fitting teflon pestle was carried out prior to centrifugation at 105,000 *g* for 1 hr. The supernatant was employed as the enzyme source for the reactions leading to sulfate activation.

Preparation of MST from rat liver. Livers from young Wistar rats, weighing about 200 g were homogenized in cold 0.15 M KCl containing 3 mM DTT to give a 20% homogenate. The 105,000 *g* cytosol was used as the enzyme source for the MST reaction without further purification.

Assay conditions for PAPS biosynthesis from sodium ^{35}sulfate. The reaction incubation mixture with a final volume of 350 μL contained the following chemicals; their final concentrations are given in parentheses: 5 μL sodium ^{35}sulfate (194 μM), 20 μL each of ATP and Mg^{2+} (8 mM) in 30 mM DTT and 50 mM glycine–NaOH buffer of pH 8.6. The reaction was started by the introduction of 15 μL of the rat skin supernatant containing about 70 μg protein. Controls were carried out with the omission of ATP or Mg^{2+}, or by the introduction of boiled enzyme solution. After 15-min incubation at 37°, the reacted mixture was boiled for 1 min in a water bath and the precipitated proteins were removed by centrifugation. Duplicate aliquots of 100 μL were removed and to each was added 20 μL of 0.5 M glucose, 5 μL hexokinase (equivalent to 10 U) followed by incubation for 30 min at 37°. This phosphorylation step by hexokinase [18] was introduced to remove the remaining ATP which would otherwise interfere in the subsequent measurement of PAPS described below.

Measurement of PAP^{35}S by coupling to the MST reaction. To each of the reaction tubes which had been subjected to the hexokinase step above was added 100 μL minoxidil (1.6 mM) and 175 μL of 50 mM KH_2PO_4–NaOH buffer at pH 7.4. The MST reaction was started by the introduction of 100 μL of a mixture containing rat liver cytosol (containing about 0.4 mg protein), 0.2 M EDTA and 0.2 M pyrophosphate (PP_i) in a ratio of 1:1:1 (by vol.). The rationale for the addition of EDTA and PP_i was to inhibit PAPS generation by the enzymes present in the rat liver cytosol; this has been explained previously [19]. The removal of ATP by the hexokinase reaction was also crucial in arresting completely the formation of PAPS by the rat liver MST. After a 10-min incubation at 37°, 1 mL ethyl acetate was added followed by 0.4 mL of 1 M ammonium hydroxide. Minoxidil ^{35}sulfate present in 0.2 mL organic phase was quantified by counting the radioactivity in 5 mL Universol scintillation fluid, according to the procedure of Johnson and Baker [20].

Quantification of PAP^{35}S. The amount of PAP^{35}S generated *in vitro* was determined by extrapolating from a standard curve of minoxidil ^{35}sulfate produced by 2.2–450 pmol PAP^{35}S added to the MST reaction mixture under identical assay conditions. ADP (produced by the hexokinase reaction in our assay) has been reported, among other nucleotides to inhibit the ST reaction [21]. In view of this, 1.6 mM ADP–Mg^{2+} was routinely added to the MST assay for the preparation of the PAPS standard curve. This concentration represents the maximum concentration of ADP–Mg^{2+} that would be carried over to the MST assay if ATP–Mg^{2+} present initially in the assay incubate of the PAPS-generating step was quantitatively converted. It must be emphasized that the radioactivity of minoxidil ^{35}sulfate formed in the experimental tubes was derived from sodium ^{35}sulfate while those measured in the standards originated from PAP^{35}S. The specific radioactivities of these ^{35}S-labeled precursors were different and this must be taken into consideration in the calculations.

Protein determination. The protein contents of the enzyme extracts were measured by the method of Bradford [22] using the Bio-rad dye with BSA as standard.

Assay conditions for PST activity of rat skin measured with different substrates. The following study serves to screen for PST activity in rat skin cytosol using conventional "M" and "P" substrates of human PST. No quantitative comparison will be made as optimal conditions developed previously for different assay systems were adopted without modification [23–25]. Ethanol present in the commercial preparation of PAP^{35}S was removed by evaporation under a stream of oxygen-free nitrogen gas. A volume of 5 μL PAP^{35}S representing a final concentration of 0.15 or 0.75 μM was routinely employed. To this was added one of the following substrates: 1 μM *p*-nitrophenol, 50 μM dopamine, 70 μM NADA or harmol. When dopamine was used, *trans*-2-phenylcyclopropylamine at a final concentration of 1 mM was preincubated with the enzyme extract to inhibit monoamine oxidase [26] as this enzyme has been reported to be present in human skin fibroblasts [27]. Controls were carried out by omitting the respective substrates or by the introduction of boiled enzyme extracts. The reaction mixture was incubated at 37° for 30 min and terminated by boiling for 1 min. The supernatant obtained after centrifugation was filtered through a membrane of 0.45 μm pore size and analysed by HPLC-radiometric procedures, following essentially the mobile systems reported previously with minor modifications. For easy reference, the mobile solvent systems for detecting the sulfate conjugates of harmol, *p*-nitrophenol, dopamine and NADA, were respectively: solvent system **A**, 35% methanol and 50% 20 mM KH_2PO_4 with 2.5 mM sodium octylsulfate at pH 3.5 [28]; system **B**, 75 mM NaH_2PO_4, 0.35 M sodium octyl sulfonic acid, 1 μM EDTA, adjusted to pH 4.0 and 4% acetonitrile plus 11% methanol [29]; solvent system **C** was essentially the same as solvent system B with only 2% methanol; and solvent **D**, 1.2% acetic acid, 1 mM EDTA at

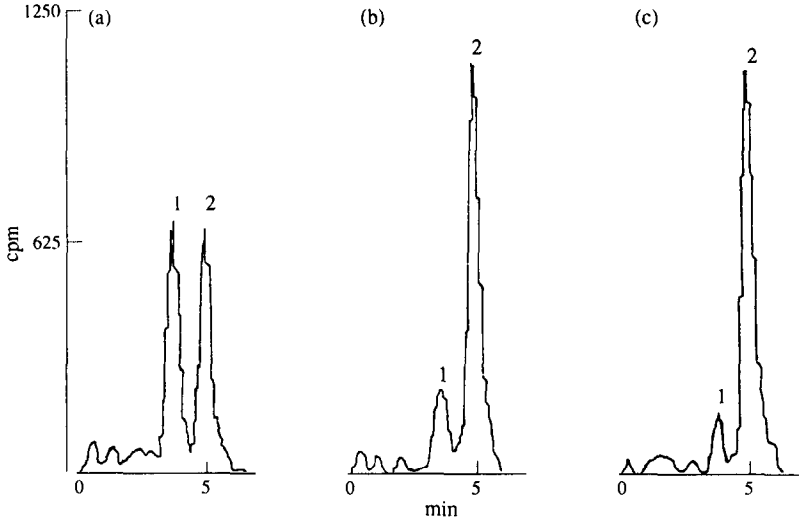

Fig. 1. HPLC chromatograms illustrating the: (a) degradation of $PAP^{35}S$ (peak 2) to $^{35}SO_4^{2-}$ and/or $AP^{35}S$ (peak 1) by rat skin or liver enzyme extract. (b) Inhibition of $PAP^{35}S$ breakdown by rat skin cytosol in the presence of 8 mM ATP–Mg^{2+} complex. An identical profile was obtained with 13.3 mM of PP_i incubated with rat liver cytosol. (c) Control incubation mixture which contained a full complement of reactants routinely employed in the PAPS-generating system except for the substitution of unlabeled sodium sulfate for the radioactive sulfate. A similar profile was observed for the control of the MST assay.

pH 4.4 and 2% methanol [30]. Mobile solvent system A was delivered at a flow rate of 0.8 mL/min and systems B and C were delivered at 1.0 mL/min through a column of 4.6 × 250 mm containing Partisil ODS of 10 μm particle size. Solvent D was delivered at 0.3 mL/min using a microbore column of 2.1 × 100 mm packed with Hypersil-ODS of 5 μm particle size.

Test for possible degradation of $PAP^{35}S$ and minoxidil ^{35}sulfate. The following sets of experiments were designed to examine if $PAP^{35}S$ generated in the assay and minoxidil ^{35}sulfate formed in the coupled MST reaction are degraded by the respective enzyme extracts, namely rat skin and rat liver cytosols. It was necessary to examine for possible degradation of $PAP^{35}S$ to SO_4^{2-} and/or $AP^{35}S$ in our assay procedure by both the skin and liver cytosols. $PAP^{35}S$ (0.26 μM) was incubated for 15 min at pH 8.6 with unlabeled Na_2SO_4, ATP–Mg^{2+} complex and 15 μL rat skin cytosolic extract, in concentrations routinely used in the PAPS-generating assay. Likewise, 0.18 μM $PAP^{35}S$ was incubated for 10 min with ADP–Mg^{2+} complex and 100 μL of a mixture containing rat liver extract, 0.2 M PP_i and 0.2 M EDTA, in a ratio of 1:1:1 (by vol.) at pH 7.4. It is apparent that these parameters mimic our standard assay conditions. Controls were simultaneously carried out without enzyme or with boiled enzyme extract. At the end of the incubation, the amount of remaining $PAP^{35}S$ and any radiolabeled products released from it were identified and quantified by HPLC-radiometry using a 4.6 × 250 mm column packed with Partisil ODS of 10 μm particle size. The flow rate of the mobile phase E which was set at 1 mL/min consisted of 3 mM tetrabutylammonium perchlorate and 30 mM KH_2PO_4 in methanol–H_2O (1:3 v/v) at pH 7.0 [31].

4-MUS was used to examine for sulfatase activity in the rat liver and skin cytosols by monitoring continuously the release of 4-MU following the procedure developed for APS-kinase [32], but in the reverse direction. A range of pH from 6.6 to 9.4 was tested with 15 μL rat skin extract at 0.15 mM 4-MUS. Likewise, sulfatase activity was also examined at pH 7.4 in 100 μL of a mixture containing rat liver cytosol, 0.2 M PP_i and 0.2 M EDTA in a ratio of 1:1:1 (by vol.); this assay condition was identical to that of the MST reaction. A commercial preparation of sulfatase (Sigma, Limpets, type V) was also included as a positive control.

RESULTS

Development of assay procedure

The measurement of $PAP^{35}S$-generation in our assay was based on the determination of minoxidil ^{35}sulfate formed. There was no production of minoxidil ^{35}sulfate when ATP or Mg^{2+} was omitted or when a boiled extract of the rat skin cytosol was used in the presence of all requisite precursors. The last control was particularly important as it demonstrated the complete removal of ATP by the hexokinase reaction. Any residual ATP would be used by the PAPS-generating enzymes present in the rat liver cytosol which was employed essentially for its MST activity in our assay procedure. The introduction of PP_i and EDTA probably also contributed to arrest completely the active PAPS-generating enzymes present in the rat liver cytosolic extract. $AP^{35}S$, liberated by the action of alkaline

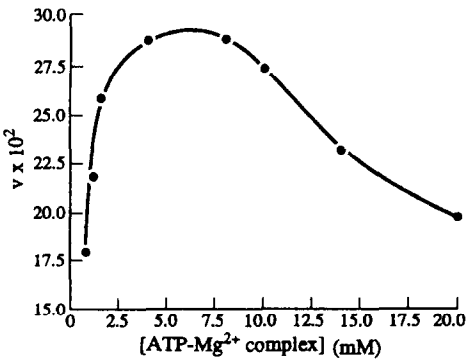

Fig. 2. Generation of $PAP^{35}S$ in rat skin cytosol from sodium ^{35}sulfate in the presence of equimolar concentrations of ATP and Mg^{2+}. The velocity (v) was expressed in nmol $PAP^{35}S$/min/mg protein.

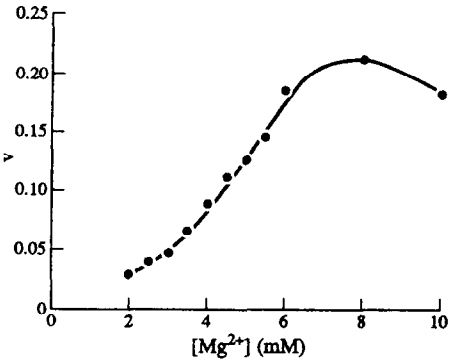

Fig. 3. Effect of Mg^{2+} concentration on the velocity (v) of $PAP^{35}S$ generation (expressed in nmol $PAP^{35}S$/min/mg protein) measured at 8 mM ATP.

phosphatase or 3′-nucleotidase on $PAP^{35}S$, co-chromatographed with authentic $^{35}SO_4^{2-}$ with a retention time of 3.8 min in solvent system **E**. Authentic $PAP^{35}S$, with a retention time of 5.0 min was readily separable from $AP^{35}S$ and $^{35}SO_4^{2-}$ (Fig. 1a). However, the last two named compounds had similar mobility and could not be resolved. About 50% of the added $PAP^{35}S$ was degraded when incubated with the rat skin at pH 8.6 or rat liver cytosol at pH 7.4 (Fig. 1a). This showed that both enzyme extracts contained hydrolytic activity toward $PAP^{35}S$. However, the inclusion of 8 mM ATP–Mg^{2+} (this being the concentration employed routinely in the PAPS-generating step) completely arrested the degradation of $PAP^{35}S$ by enzymes present in the rat skin cytosol. Likewise, 13.3 mM PP_i added to the standard MST-coupled assay was effective in preventing enzymatic degradation of PAPS by the rat liver cytosol (Fig. 1b). The protective action of ATP and PP_i was apparent from the quantitative recovery of $PAP^{35}S$ as compared to the corresponding controls (Fig. 1c). The commercial preparation of $PAP^{35}S$ on prolonged storage showed a minor degradation product, with a retention time similar to inorganic ^{35}sulfate and $AP^{35}S$.

Kinetic data of PAPS generation

The pH optimum for the generation of PAPS in rat skin cytosol was 8.6. This was similar to that observed in human liver [33]. The reaction increased linearly for incubation up to 15 min and with enzyme extracts containing up to 70 μg protein. ATP and Mg^{2+} present in equimolar concentrations in the assay incubate was inhibitory above 8 mM (Fig. 2). The effect of varying Mg^{2+} in the presence of 8 mM ATP is illustrated in Fig. 3. As the ATP concentration was fixed at 8 mM, the peak activity observed at 8 mM Mg^{2+} suggests a 1:1 stoichiometry between the concentrations of ATP and Mg^{2+}. Analysis of the kinetic data by the Enzpack programme [34] showed that the apparent K_m for ATP–Mg^{2+} was 1 mM (Fig. 4a) and that for sodium ^{35}sulfate was 68 μM (Fig. 4b). Excess or free ATP not complexed with Mg^{2+} was a competitive inhibitor with an

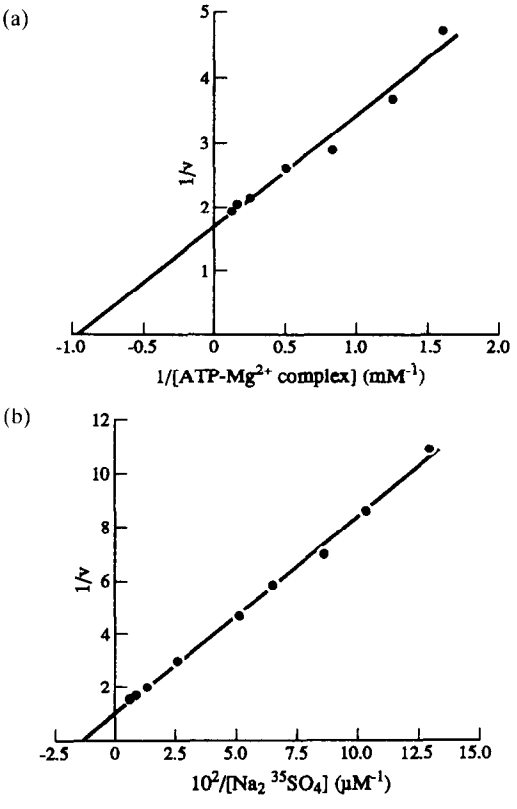

Fig. 4. Lineweaver–Burk plots of $PAP^{35}S$-generating activity in rat skin where the velocity (v) was expressed in nmol $PAP^{35}S$/min/mg protein against: (a) 0.6–8 mM of ATP–Mg^{2+} complex measured at 39 μM sodium ^{35}sulfate, (b) 7.8–155 μM sodium ^{35}sulfate measured at 8 mM ATP–Mg^{2+} complex.

apparent K_i of 0.32 mM derived from the secondary plot (Fig. 5, inset).

PAPS generated was extrapolated from a standard curve obtained with 2.2–450 pmol $PAP^{35}S$ which

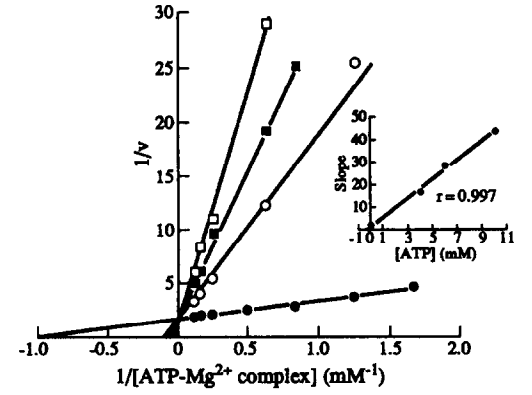

Fig. 5. Inhibition of PAPS generation by excess (free) ATP of 4 mM (○), 6 mM (■) and 10 mM (□). The velocity (v) expressed in nmol $PAP^{35}S$/min/mg protein, was obtained for substrate concentrations of 0.6–8 mM ATP–Mg^{2+} complex. Secondary plot derived from the above data is shown in the inset.

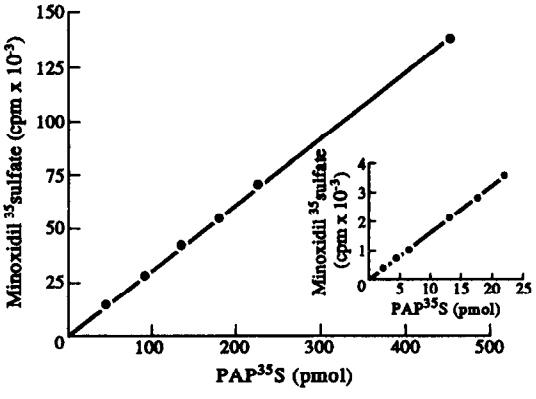

Fig. 6. Standard curve of $PAP^{35}S$ assayed by the coupled minoxidil sulfotransferase reaction. Linearity was also observed when <25 pmol $PAP^{35}S$ were used (see inset).

Table 1. Separation and identification of $PAP^{35}S$ and the radiolabeled sulfoconjugates of harmol, *p*-nitrophenol, dopamine and NADA formed by rat skin cytosol by HPLC-radiometry

Substrate	Retention time (min) $PAP^{35}S$	Sulfate conjugate	Mobile system*
Harmol	3.8	7.1	A
p-Nitrophenol	2.3	6.3	B
Dopamine	3.3	5.2	C
NADA	3.3	7.8	D

* As described in text.

showed a linear relationship with minoxidil ^{35}sulfate formed (Fig. 6). There was no evidence of sulfatase activity on 4-MUS in the rat skin (measured between pH 6.6 and 9.4) and in rat liver extracts (measured at pH 7.4). Sulfatase activity on 4-MUS was however demonstrable by the liberation of 4-MU at pH 5 using a commercial preparation of sulfatase. At this acidic pH, optimal for hydrolases, the rat liver extract showed low but detectable sulfatase activity.

Under the optimum conditions of assay, the specific activities of PAPS-generation by rat skin and liver cytosols were, respectively, 0.15 and 1.16 nmol $PAP^{35}S$/min/mg protein. The activity in the rat skin is about 13% of that of the corresponding liver.

Sulfate conjugation of prototype substrates by rat skin extract

PST activity in rat skin extract was also studied with dopamine, NADA, *p*-nitrophenol and harmol using $PAP^{35}S$. Radiolabeled sulfate conjugates formed were separated from $PAP^{35}S$ and identified by HPLC-radiometric procedures. The chromatographic data obtained are shown in Table 1. No radioactive product was formed in control incubates where the sulfate acceptor was omitted or when boiled enzyme was used in the assay. Two pH optima of 7.0 and 9.0 were apparent with NADA, while dopamine exhibited a pH optimum of 9.4. These observations were similar to those reported earlier with rat brain [35]. A biphasic response was also observed for *p*-nitrophenol-PST at pH 6.6 and 8.6, while harmol-PST showed a broad pH activity curve from pH 6.6 to 7.8.

DISCUSSION

A radiometric method was developed for the measurement of PAPS-generation in rat skin cytosol by quantifying minoxidil ^{35}sulfate formed in a reaction catalysed by rat liver MST. Several other sulfate acceptor substrates namely harmol [36], 4-methylumbelliferone [33, 37] and NADA [33] have been used for this purpose in similar PST-coupled reactions. However, these methods were not sensitive for tissues whose PAPS-generating activities are low. From our study of the kinetics of MST [13], it was apparent that rat liver MST could provide a more sensitive assay procedure. The simplicity and reproducibility of the one-step ethyl acetate extraction procedure developed by Johnson and Baker [20] was an added attractive consideration as many samples could be accommodated. Indeed, a comparison of the standard curve of PAPS showed that as little as 2 pmol PAPS (Fig. 6) were detectable compared to 250 pmol used in our earlier HPLC-electrochemical and fluorimetric procedures [33]. This could be translated into an increase in sensitivity of 100-fold, which made it possible to measure PAPS biosynthesis *in vitro* by skin and brain (unpublished data) extracts. A further modification of our procedure was the addition of hexokinase to quantitatively remove ATP added to the PAPS-generating assay incubation mixture.

Like most other biochemical reactions involving ATP, the ATP–Mg^{2+} complex was shown to be the

substrate for PAPS biosynthesis in rat skin extract. Free ATP, in excess of total Mg^{2+} was a competitive inhibitor (Fig. 5). Both the above phenomena had been observed with the two enzymes of sulfate activation, namely ATP sulfurylase [38] and APS kinase [39] purified from *Penicillium chryogenium*. Competitive inhibition was also demonstrated in the latter instance. It is interesting that similar kinetics were shared by such diverse organisms and demonstrable using purified enzymes [38, 39] and relatively crude skin extracts.

PAPS-degrading enzymes are present in various subcellular fractions and widely distributed in mammalian tissues [17, 40, 41]. Their presence in the enzyme extracts of rat skin and liver used in our assay would affect the measurement of sulfate-activating activity. Fortuitously, ATP added to the PAPS-generating system and PP_i, introduced routinely to the coupled-MST reaction principally for its inhibitory action on ATP sulfurylase as discussed [19], were shown to be effective inhibitors of sulfohydrolytic and/or phosphohydrolytic activities in the rat skin and liver cytosols. These two phosphates had previously been found to inhibit hydrolysis of PAPS and APS in mammalian tissues [40–43].

Besides minoxidil [13], rat skin cytosol was also shown to possess enzyme activity capable of sulfating a number of common substrates of PST, namely harmol, *p*-nitrophenol, dopamine and NADA. No quantitative comparison was made as the PST procedures differed in assay conditions for the different substrates with respect to their concentrations and pH. Both prototype "M" and "P" substrates of human PST were found to be sulfoconjugated. These reactions would employ the "active sulfate" formed *in situ*. The cutaneous tissue is constantly subjected to xenobiotic insults in the form of environmental pollutants, cosmetics and topically applied drugs. The demonstration of PAPS generation and PST activity in rat skin in this study suggests that this organ is metabolically active in sulfate conjugation and would contribute to the detoxification of foreign compounds before their entry into the systemic circulation. The K_m value for inorganic sulfate of 68 μM is below the concentration of inorganic sulfate of 0.9 mM in rat serum [44]. If transport of sulfate to the skin is rapid as suggested by the observation with human skin fibroblasts [45], the concentration of inorganic sulfate is unlikely to be limiting in the formation of PAPS.

In contrast to detoxification of xenobiotics, sulfate conjugation in the skin could increase the bioreactivity of foreign compounds as illustrated in the sulfation of minoxidil [9] and 5-hydroxymethylchrysene [46]. Together with *N*-acetyltransferase which has been reported to be present in skin [47], PST and PAPS generation may contribute to the carcinogenic activity of arylamines.

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