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INHIBITION OF RAT LIVER HYDROXYSTEROID SULFOTRANSFERASE ACTIVITY BY ALKYLAMINES

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Abstract—Triethylamine, which was used as an elution solvent for column chromatography to purify chemically synthesized 3′-phosphoadenosine 5′-phosphosulfate (PAPS), was a potent inhibitor of rat liver sulfotransferase (ST) activities toward androsterone and dehydroepiandrosterone, but not ST activities toward cortisol and 2-naphthol. Examination of fourteen primary, secondary and tertiary amines revealed that a secondary amine, di-*n*-butylamine, and three tertiary amines, triethylamine, tri-*n*-propylamine and tri-*n*-butylamine, specifically inhibited ST activities toward androgen.

Sulfotransferase (ST)† catalyzes the transfer of a sulfate group from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to a wide range of xenobiotics and endogenous compounds [1–3]. Evidence that there are several ST isoenzymes in rat liver cytosols has been accumulating [1–3]. Special inhibitors are valuable molecular tools for the study of the properties and multiplicities of ST isoenzymes with wide and overlapping substrate specificities. The ST activity can be inhibited by substrates and their analogues. Pentachlorophenol [4], 2,6-dichloro-4-nitrophenol [4], aryl carboxylic acids [5], benzo[*a*]pyrene dihydrodiols [6] and benzylic alcohols [7] are known to be strong inhibitors of aryl ST. Some 2- and 4-substituted estra-1,3,5(10)-trien-17β-ols strongly inhibit estrone ST activity [8]. Adenosine 3′,5′-bisphosphate (PAP), an end product of PAPS, and several PAP and ATP analogues are powerful inhibitors of ST isoenzymes [9]. It is known that several drugs inhibit rat liver ST activity toward cortisol [10] and human liver ST activities toward dehydroepiandrosterone and estrone [11]. Several potent ST inhibitors appear to be present in tissue extracts [12] and in food and red wine [13], but little is known of their structures.

PAPS has been prepared either chemically [14–16] or biochemically [17]. During our studies on rat liver aryl and hydroxysteroid ST isoenzymes, we have used biochemical PAPS as a coenzyme. Recently we prepared PAPS by chemical means and compared its sulfate donor ability with that of biochemical PAPS in rat liver cytosols. To our surprise, chemical PAPS could sulfate androgens such as androsterone and dehydroepiandrosterone only at a much lower rate than biochemical PAPS. However, the chemical PAPS sulfated cortisol, 4-nitrophenol and 2-naphthol at a rate similar to that of biochemical PAPS. In this paper, we report that triethylamine used as an elution solvent for column chromatography to purify chemical PAPS specifically inhibited androgen sulfation. Among fourteen primary, secondary and tertiary amines examined thus far, the tertiary amines with 2 to 4 carbon atom side chains are a potent and specific inhibitor of the hydroxysteroid ST activity.

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† Abbreviations: ST, sulfotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; and PAP, adenosine 3′,5′-bisphosphate.

MATERIALS AND METHODS

Materials

[1,2,6,7-^{3}H]Dehydroepiandrosterone (100 Ci/mmol), [9,11-^{3}H]androsterone (60 Ci/mmol) and [4-^{14}C]cortisol (55 mCi/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). DEAE-cellulose DE-52 was obtained from Whatman Biosystems (Maidstone, Kent, U.K.). PAPS, DEAE-Sephadex A-25 and Sephadex G-10 were purchased from Pharmacia (Uppsala, Sweden). 2-Naphthyl sulfate (potassium salt), PAP and Dowex 1 were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Crowned dinitrophenylazophenol (barium salt) was donated by Dr. Kenichiro Nakashima, Nagasaki University. All other reagents were of the highest grade available.

Animals and preparation of cytosolic fractions

Adult male and female rats of the Wistar strain were obtained from Sankyo Lab. (Tokyo, Japan). The animals had free access to food and water and were decapitated. A 25% (w/v) liver homogenate was prepared in buffer A (0.25 M sucrose and 0.1 M Tris–HCl buffer, pH 7.4) for the assay of ST activity or buffer B (0.25 M sucrose, 3 mM 2-mercaptoethanol, 0.1 mM EDTA and 10 mM Tris–HCl, pH 7.4) for column chromatography. Cytosolic fractions were obtained by differential centrifugation (2000 *g* for 10 min, 16,000 *g* for 45 min, and 105,000 *g* for 60 min).

Enzyme assay

ST activities toward dehydroepiandrosterone, androsterone, cortisol and 2-naphthol were assayed by a slight modification of a method described

Table 1. Sulfate donor abilities of various PAPS preparations in rat liver cytosols

Substrate	Coenzyme	ST activity (nmol/min/mg) Male	Female
NAP	Biochemical PAPS	0.63 ± 0.09 (1.00)	0.41 ± 0.08 (1.00)
	Chemical PAPS	0.70 ± 0.08 (1.11)	0.51 ± 0.06 (1.24)
	Commercial PAPS	0.75 ± 0.16 (1.19)	0.41 ± 0.15 (1.00)
CS	Biochemical PAPS	0.03 ± 0.01 (1.00)	0.08 ± 0.02 (1.00)
	Chemical PAPS	0.04 ± 0.01 (1.33)	0.10 ± 0.02 (1.25)
	Commercial PAPS	0.03 ± 0.01 (1.00)	0.07 ± 0.03 (0.88)
AD	Biochemical PAPS	0.13 ± 0.02 (1.00)	0.28 ± 0.04 (1.00)
	Chemical PAPS	0.04 ± 0.01* (0.31)	0.09 ± 0.02* (0.32)
	Commercial PAPS	0.13 ± 0.03 (1.00)	0.29 ± 0.05 (1.04)
DHA	Biochemical PAPS	0.20 ± 0.05 (1.00)	0.45 ± 0.07 (1.00)
	Chemical PAPS	0.08 ± 0.02† (0.40)	0.17 ± 0.04* (0.38)
	Commercial PAPS	0.21 ± 0.04 (1.05)	0.41 ± 0.05 (0.91)

Each value is the mean ± SD for 3 animals. Values in parentheses indicate the ST activity relative to that assayed with biochemical PAPS as a coenzyme. Abbreviations: NAP, 2-naphthol; CS, cortisol; AD, androsterone; and DHA, dehydroepiandrosterone.

Significantly different from ST activity assayed with biochemical PAPS as a coenzyme: *$P < 0.01$, and †$P < 0.05$.

previously [18, 19]. In brief, the standard incubation medium contained 10 mM $MgCl_2$, 100 μM PAPS, 100 μM EDTA and 50 μM [^{3}H]dehydroepiandrosterone (0.01 μCi), 86 μM [^{3}H]androsterone (0.01 μCi), 40 μM [^{14}C]cortisol (0.006 μCi) or 0.5 mM 2-naphthol and 0.1 M Tris–HCl buffer, pH 7.4, in a total volume of 0.50 mL. The incubation was carried out at 37° for 10–20 min. The blank values were obtained from control incubations in which PAPS was omitted. For kinetic studies, the assay was identical with the standard incubation except that different sets of substrate concentrations (40–160 μM PAPS and 50 μM dehydroepiandrosterone or 1–3 μM dehydroepiandrosterone and 100 μM PAPS) were used in the presence of 0, 100 or 300 μM triethylamine as a ST inhibitor. The incubation was carried out at 37° for 7 min.

Protein concentrations were determined by the method of Bradford [20] with bovine serum albumin as the standard.

DEAE-cellulose chromatography

Cytosolic fractions obtained from three female rat livers were fractionated on a DEAE-cellulose column (2.5 × 70 cm) as described previously [21, 22]. The column was washed with buffer B and eluted with a linear 0 to 0.2 M NaCl gradient in buffer B (3000 mL). ST activities were divided into four fractions (Fr. 1 to Fr. 4), according to ST activities toward 2-naphthol, cortisol and dehydroepiandrosterone.

Preparation of PAPS

Biochemical synthesis. PAPS was prepared according to the method of Singer [17]. In brief, the precipitates obtained by 34–46% saturation of ammonium sulfate of female rat liver supernatant were incubated in the presence of ATP to yield PAPS. Crude PAPS was subjected to chromatography on a Dowex 1 column and eluted with 0.3 to 1.1 M NaCl gradient in 1 mM Tris–HCl buffer, pH 8.7. After concentration of the PAPS fraction, NaCl was removed by Sephadex G-10 column chromatography by elution with 1 mM Tris–HCl buffer, pH 8.7. The purified PAPS was more than 98% pure, as judged by HPLC [23], and was stored at −80° as a 1 mM solution until used.

Chemical synthesis. Adenosine 2′,3′-cyclic phosphate-5′-phosphosulfate was prepared by reaction of adenosine with pyrophosphoryl chloride [24], followed by reaction with triethylamine-*N*-sulfonic acid [16]. PAPS was prepared by reaction of adenosine 2′,3′-cyclic phosphate 5′-phosphosulfate with ribonuclease T_2 and purified by DEAE-Sephadex A-25 column chromatography by elution with a linear gradient of 0.05 to 1 M triethylammonium bicarbonate, pH 7.5 [15]. The solvent of the purified PAPS fraction was removed *in vacuo*, the pH was adjusted to 7 with ammonia water, and the solvent was evaporated repeatedly as described previously [15]. The purified PAPS was more than 98% pure, as judged by HPLC [23], and was stored at −80° as a 1 mM solution until used.

Assay of triethylamine contents in the purified chemical PAPS preparation

The content of triethylamine in the chemical PAPS preparation was determined according to a slight modification of the method described by Nakashima *et al.* [25]. To 0.20 mL of the sample (1 mM PAPS preparations were diluted 5–8 times with water) or standard triethylamine solution were added 0.10 mL of 0.1 N NaOH and 0.30 mL of diethyl ether. After vigorous shaking and subsequent centrifugation at 1500 *g* for 5 min, 0.20 mL of the ethereal layer was taken and mixed with 4 mL of methanol solution

Table 2. Sulfate donor abilities of various PAPS preparations in partially purified female rat liver ST fractions

Substrate	Coenzyme	Relative ST activity			
		Fr. 1	Fr. 2	Fr. 3	Fr. 4
NAP	PAPS(BI)	1.00 (1.97)	1.00 (2.48)	1.00 (3.33)	1.00 (4.90)
	PAPS(CH)	1.09	1.05	1.09	1.21
	PAPS(BI) + 100 μM TEA	1.01	1.00	1.02	1.06
	PAPS(BI) + 300 μM TEA	0.99	0.98	1.05	1.06
CS	PAPS(BI)	1.00 (0.72)	1.00 (0.24)	1.00 (0.10)	0
	PAPS(CH)	1.24	1.25	1.30	0
	PAPS(BI) + 100 μM TEA	0.90	1.02	0.94	ND
	PAPS(BI) + 300 μM TEA	1.04	0.98	0.96	ND
AD	PAPS(BI)	1.00 (2.70)	1.00 (1.20)	1.00 (0.78)	1.00 (0.29)
	PAPS(CH)	0.12	0.07	0.07	0
	PAPS(BI) + 100 μM TEA	0.47	0.41	0.47	ND
	PAPS(BI) + 300 μM TEA	0.23	0.21	0.16	ND
DHA	PAPS(BI)	1.00 (4.67)	1.00 (2.08)	1.00 (0.92)	1.00 (0.30)
	PAPS(CH)	0.11	0.09	0.11	0.13
	PAPS(BI) + 100 μM TEA	0.35	0.41	0.49	ND
	PAPS(BI) + 300 μM TEA	0.15	0.15	0.25	ND

After DEAE-cellulose chromatography, ST fractions were divided into four fractions (Fr. 1–Fr. 4) as described in Materials and Methods. A 100 or 300 μM concentration of triethylamine (TEA) was added to the assay medium containing biochemical PAPS (PAPS(BI)) as a coenzyme to determine the inhibitory effect of TEA. Each value represents the ST activity relative to that assayed with PAPS(BI) as a coenzyme and is the mean of two separate determinations. Values in parentheses indicate ST activity expressed as nmol/min/mg. PAPS(CH) = chemical PAPS. ND = not determined. Other abbreviations are the same as in Table 1.

containing 50 μM crowned dinitrophenylazophenol and 50 μM $BaCl_2$, and the absorbance was measured at 520 nm. Blank values were obtained by using 0.2 mL of water instead of the sample.

RESULTS

Comparison of sulfate donor ability of various PAPS preparations

Purities of biochemical and chemical PAPS preparations were more than 98%, although commercial PAPS was about 80% pure. It was confirmed that these PAPS preparations did not contain a potent ST inhibitor, PAP. Table 1 shows the comparison of sulfate donor abilities of biochemical, chemical and commercial PAPS preparations, determined by sulfation of 2-naphthol, cortisol, androsterone and dehydroepiandrosterone in male and female rat liver cytosols. ST activity toward 2-naphthol was higher in males, whereas ST activities toward cortisol, dehydroepiandrosterone and androsterone were higher in females as reported previously [1–3, 18, 19]. Sulfate donor abilities to 2-naphthol and cortisol were virtually identical among biochemical, chemical and commercial PAPS preparations in both sexes, whereas chemical PAPS gave only 30–40% abilities of biochemical or commercial PAPS preparations in the sulfation of dehydroepiandrosterone and androsterone irrespective of sex (Table 1).

We prepared partially purified ST fractions by DEAE-cellulose chromatography of female rat liver cytosols, and ST activities were divided into four fractions. Fractions 1 and 2 showed comparatively high ST activities toward androsterone, dehydroepiandrosterone and cortisol, whereas Fractions 3 and 4 showed comparatively high ST activities toward 2-naphthol (Table 2). Chemical PAPS showed only 7–15% sulfate donor abilities toward androsterone and dehydroepiandrosterone as compared with that of biochemical PAPS, while both PAPS preparations showed virtually identical rate of sulfation toward 2-naphthol and cortisol (Table 2). It should be noted that chemical PAPS provided much lower sulfate donor abilities toward androgen in partially purified ST fractions than in cytosolic fractions.

Triethylamine bicarbonate buffer was used as an elution solvent for column chromatography to purify chemical PAPS. Therefore, the effect of triethylamine on ST activities was examined by the addition of 100 or 300 μM triethylamine to the assay medium containing biochemical PAPS as a coenzyme. It became evident that triethylamine inhibited ST activities toward dehydroepiandrosterone and androsterone, but not ST activities toward 2-naphthol and cortisol (Table 2). Subsequently, we assayed triethylamine contents in the chemical PAPS preparations.

Triethylamine as a specific inhibitor of hydroxysteroid ST activity

The contents of triethylamine in chemical PAPS preparations were determined by the crowned

dinitrophenylazophenol method [25]. It became apparent that 1 mM chemical PAPS preparations contained 1.8 to 5.9 mM triethylamine, indicating that 180 to 590 μM triethylamine should be present in the assay medium. It was found out that the assay medium used in Tables 1 and 2 included 440 μM triethylamine. When chemical PAPS preparations containing 180, 440 and 590 μM triethylamine were incubated with dehydroepiandrosterone as substrate in rat liver cytosols, their relative sulfate donor abilities were 62 ± 10, 44 ± 7 and 27 ± 9%, respectively, as compared with that of biochemical PAPS. For comparison, biochemical PAPS was incubated in the presence of 180, 440 and 590 μM triethylamine and their relative abilities were 58 ± 8, 43 ± 6 and 31 ± 5%, respectively. Good coincidence was observed between the contents of triethylamine and the relative sulfate donor abilities of chemical and biochemical PAPS preparations. These results provide evidence that triethylamine present as a contaminant in chemical PAPS preparations specifically inhibited ST activities toward androgen.

Inhibition of hydroxysteroid ST activity by various amines

To extend structure–activity relationships of *in vitro* inhibitors of hydroxysteroid ST activity, we examined fourteen primary, secondary and tertiary amines for inhibitory actions on ST activities toward dehydroepiandrosterone, cortisol and 2-naphthol. Table 3 shows the effects of 300 μM amines on ST activities of male and female rat liver cytosols. A secondary amine, di-*n*-butylamine and three tertiary amines, triethylamine, tri-*n*-propylamine and tri-*n*-butylamine, inhibited dehydroepiandrosterone ST activity by 40–60% irrespective of sex; however, 2-naphthol and cortisol ST activities were not affected significantly by these amines (Table 3).

To study the nature of the inhibition of hydroxysteroid ST activity by these amines, we used partially purified hydroxysteroid ST fraction corresponding to Fraction 1 as an enzyme source and triethylamine as a ST inhibitor. Lineweaver–Burk plots indicate that the data of the inhibition by triethylamine fit a noncompetitive inhibition. Apparent K_m and K_i values for dehydroepiandrosterone were 20.4 ± 1.1 and 165 ± 23 μM, respectively, while apparent K_m and K_i values for PAPS were 52.6 ± 11.4 and 177 ± 44 μM, respectively. Marcus *et al.* [26] purified the major hydroxysteroid ST (hydroxysteroid ST 2) from female rat liver, which appears to correspond to the partially purified ST fraction used in this study. The apparent K_m values for dehydroepiandrosterone and PAPS (with dehydroepiandrosterone as substrate) are 24 and 47 μM, respectively, which are in good accord with our data. These authors reported that PAP competitively inhibits PAPS, and the K_i value for PAPS is 14 μM. We also confirmed that PAP

Table 3. Effects of fourteen amines on ST activities in rat liver cytosols

Amine	Relative ST activity			
	Male		Female	
	DHA	NAP	DHA	CS
Control	1.00 ± 0.09 (0.09 ± 0.01)	1.00 ± 0.27 (1.18 ± 0.32)	1.00 ± 0.09 (0.61 ± 0.07)	1.00 ± 0.48 (0.16 ± 0.08)
Primary amine				
Methylamine	0.87 ± 0.15	ND	0.99 ± 0.18	0.96 ± 0.16
Ethylamine	0.79 ± 0.25	ND	1.10 ± 0.15	0.96 ± 0.21
n-Propylamine	0.96 ± 0.24	0.91 ± 0.56	1.13 ± 0.14	1.06 ± 0.10
n-Butylamine	1.03 ± 0.16	1.01 ± 0.46	1.13 ± 0.08	1.07 ± 0.12
Secondary amine				
Dimethylamine	0.83 ± 0.23	ND	0.93 ± 0.20	1.06 ± 0.16
Diethylamine	0.95 ± 0.14	0.98 ± 0.56	1.02 ± 0.18	1.09 ± 0.12
Di-*n*-propylamine	0.85 ± 0.22	1.07 ± 0.52	0.98 ± 0.15	1.04 ± 0.11
Di-*n*-butylamine	0.44 ± 0.22*	1.29 ± 0.57	0.45 ± 0.04*	1.13 ± 0.30
Tertiary amine				
Trimethylamine	0.99 ± 0.14	1.06 ± 0.47	0.95 ± 0.12	1.12 ± 0.10
Triethylamine	0.48 ± 0.09*	1.09 ± 0.56	0.52 ± 0.07*	1.21 ± 0.20
Tri-*n*-propylamine	0.55 ± 0.14*	0.98 ± 0.46	0.62 ± 0.07*	1.15 ± 0.38
Tri-*n*-butylamine	0.39 ± 0.10*	0.98 ± 0.52	0.46 ± 0.11*	1.07 ± 0.37
Tri-benzylamine	1.02 ± 0.10	1.15 ± 0.35	1.03 ± 0.21	ND
N,N-Dimethylaniline	0.98 ± 0.06	ND	1.03 ± 0.29	1.11 ± 0.12

Biochemical PAPS was used as a coenzyme. The concentration of each amine was 300 μM, and ST activities were determined as described in Materials and Methods. Each value represents the ST activity relative to that assayed with biochemical PAPS as a coenzyme and is the mean ± SD for 3–6 animals. Values in parentheses indicate ST activity expressed as nmol/min/mg. ND = not determined. Other abbreviations are the same as in Table 1.

* Relative ST activity was significantly different from that assayed with biochemical PAPS as a coenzyme ($P < 0.01$).

was a competitive inhibitor of PAPS with K_i value of 20 μM (data not shown).

DISCUSSION

PAPS has been prepared chemically [14–16] or biochemically [17] and has been used as a coenzyme for the assay of ST activities. In this report, it became evident that triethylamine, which is used as an elution solvent for the purification of chemical PAPS, is a potent inhibitor of hydroxysteroid ST. Since the amine readily forms salt with phosphate and sulfate moieties of PAPS, caution must be taken to remove it by repeated evaporation of the solvent for the assay of hydroxysteroid ST activity.

It is well known that hydroxysteroid ST activity can be inhibited by substrates and their analogues [1–3]. Trialkylamines are not substrates for hydroxysteroid ST; however, some of them specifically inhibit the enzyme activity as described in this report. It should be noted that the tertiary amines having alkyl groups with 2 to 4 carbon atom side chains strongly inhibited ST activities toward androsterone and dehydroepiandrosterone. Primary and secondary amines, except for di-*n*-butylamine, did not show any inhibitory effect on ST activities toward androgen. At present, it is not clear how these amines can interact with the special ST isoenzyme(s). Kinetic study with partially purified hydroxysteroid ST fraction indicates that triethylamine is a noncompetitive inhibitor of both dehydroepiandrosterone and PAPS. Though the study with pure enzyme should be required, these results suggest that triethylamine interacts with the allosteric portion of the enzyme probably by forming complexes with the free enzyme and enzyme–substrate complexes, resulting in inhibition of the enzyme activity. It is interesting that various drugs strongly inhibit human liver ST activities toward dehydroepiandrosterone and estrone [11]. These drugs are synthetic steroids, antisteroidals and tertiary amine drugs such as antidepressants and antihistamines. Most of the tertiary amine drugs have a complex tricyclic structure and the kinetic data appear to indicate mixed-type inhibition [11]. It is intriguing to know whether triethylamine and related amines can inhibit human liver dehydroepiandrosterone ST activity.

Recently we provided evidence that androsterone-sulfating ST consists of several pI variants with the same molecular mass of 30,000 [22]. It became apparent that the pI variants have *N*-terminal amino acid sequences [27] identical with that of an isoenzyme of hydroxysteroid ST (STa), which is active toward dehydroepiandrosterone [28]. Thus, it is not surprising that ST activities toward androsterone and dehydroepiandrosterone were similarly inhibited by these amines.

Another interesting aspect is that cortisol ST activity was not inhibited by these amines at all. It appears difficult to completely separate glucocorticoid (cortisol) ST(s) from hydroxysteroid ST(s) [1, 2]. Cortisol is a substrate of purified rat liver hydroxysteroid STs [3], whereas dehydroepiandrosterone is a substrate of purified rat liver glucocorticoid STs [2]. At present, it is not known whether these ST isoenzymes are different proteins. However, the present study provides evidence that the glucocorticoid (cortisol) ST(s) is distinct from the hydroxysteroid (dehydroepiandrosterone) ST(s).

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