Pergamon

Biochemical Pharmacology, Vol. 47, No. 10, pp. 1743–1749, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0046-N

2,6-DICHLORO-4-NITROPHENOL (DCNP), AN ALTERNATE-SUBSTRATE INHIBITOR OF PHENOLSULFOTRANSFERASE

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(*Received* 16 *August* 1993; *accepted* 21 *January* 1994)

Abstract—2,6-Dichloro-4-nitrophenol (DCNP)-^{35}sulfate was identified and quantified by an HPLC–radiometric assay following its biosynthesis *in vitro* from ^{35}S-labeled 3′-phosphoadenosine-5′-phosphosulfate (PAP^{35}S) by phenolsulfotransferase (PST) of rat liver cytosol. Acid hydrolysis of DCNP-^{35}sulfate produced almost stoichiometric release of inorganic ^{35}sulfate and DCNP. In two-substrate experiments of sulfation of *p*-nitrophenol (*p*-NP) or dopamine (prototype substrates for P and M human PST forms), 10 μM DCNP inhibited the reactions by about 15 and 78%, respectively. This contrasts with its action on PST of human origin where the P-PST was more sensitive to DCNP inhibition. In all mixed bi-substrate experiments, a reciprocal relationship between the two sulfated products was observed. Kinetic data showed that *p*-NP inhibited the sulfation of DCNP competitively. Likewise the sulfation of *p*-NP and dopamine was competitively inhibited by DCNP. However, non-competitive inhibition was observed in the sulfation of *p*-NP by DCNP, measured at varying concentrations of PAP^{35}S. The above kinetic data suggest that DCNP is an alternate-substrate inhibitor of rat liver PST.

Key words: DCNP; phenolsulfotransferase (PST); rat liver; dopamine

DCNP† has been shown to completely inhibit the sulfate conjugation of harmol *in vivo* and *in vitro* in rat liver [1, 2]. It has also been used extensively to distinguish the two forms of PST of human origin [3, 4] such as the liver [5], platelets [6], intestine [7], brain [8] and pituitary [9]. The P form of human PST catalyses the sulfation of simple phenols such as *p*-NP and was significantly inhibited at micromolar concentration of DCNP while millimolar concentration elicited only 5–10% inhibition of the M form [3, 4]. Though human PST forms could be classified by differential DCNP inhibition, it is not applicable to PST of rat tissues because of the presence of multiple PST isoforms [10–12]. In this communication, we present evidence for the biosynthesis and characterization of DCNP-sulfate, and examined the action of DCNP on PST of rat liver cytosol. Based on kinetic data obtained, it was proposed that DCNP acts as an alternate-substrate inhibitor.

MATERIALS AND METHODS

Chemicals. *p*-NP, dopamine, sodium 1-octane sulfonic acid, *trans*-2-phenylcyclopropylamine, nialamide, PAPS and β-glucuronidase (containing sulfatase activity) from *Helix pomatia* (G0751) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). PAP^{35}S (2.5, 1.92 and 1.5 Ci/mmol of 98% radionuclide purity) and sodium ^{35}sulfate (758.0 mCi/mmol of >99% radionuclide purity) were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). 2,6-Dichloro-4-nitrophenol (>98% pure) was purchased from Fluka Chemie AG (Switzerland). Methanol (HPLC grade) was from Fisher Scientific (NJ, U.S.A.). Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany).

Preparation of rat liver cytosol. A 20% (w/v) homogenate of rat liver was prepared in 0.15 M KCl containing 3 mM dithiothreitol. The high-speed supernatant obtained after centrifugation at 108,000 *g* for 30 min at 4° was stored in small aliquots at −80°.

Assay of PST activity. Single-substrate experiments. These were carried out with the following acceptors: DCNP (6.7 μM to 1 mM), *p*-NP (0.05 to 5 μM) and dopamine (0.5 to 500 μM) in the presence of 0.15 μM PAP^{35}S, using 30 μL of rat liver cytosol containing 300–400 μg protein. 50 mM KH_2PO_4–NaOH buffer at pH 8.0 was added to a final volume of 150 μL. This pH was chosen as preliminary experiments had shown that it was optimum for the sulfation of DCNP although it deviated from the optima of 6.5 and 9.0, commonly employed for the sulfation of *p*-NP and dopamine [13–15]. Whenever dopamine was present in the assay, a monoamine oxidase inhibitor such as *trans*-2-phenylcyclopropylamine or nialamide was added to the enzyme extract at a final concentration of 1 mM. The reaction was stopped after a 30 min incubation by boiling for one minute. After centrifugation and filtration, 5 to 25 μL of the supernatant was injected for HPLC–radiometric analysis.

For the sulfation of harmol, the assay mixture contained 70 μM harmol and 33 μM unlabeled PAPS

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† Abbreviations: DCNP, 2,6-dichloro-4-nitrophenol; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PST, phenolsulfotransferase; ST, sulfotransferase; *p*-NP, *p*-nitrophenol; DAD, diode-array detection.

together with the other components stated above. The reaction was terminated by adding 25 μL each of 5% zinc sulfate, 0.3 M barium hydroxide and 0.3 M barium acetate. After centrifugation, 170 μL of the supernatant was transferred to another series of tubes. Unreacted harmol was extracted twice with 340 μL ethyl acetate after the pH was adjusted to 9.5 with 0.2 M NaH_2PO_4. The aqueous layer, which contained harmol sulfate was filtered and 10 μL was injected for HPLC–fluorimetric analysis following the procedure of Tan *et al.* [16].

Mixed-substrate experiments. The assay conditions were identical to the above except that the two substrates were present in various combinations of concentrations together with the corresponding controls where the second-mentioned co-substrate was omitted as shown below:

(i) 0.17 to 3.3 μM *p*-NP in the presence of 33, 67 or 100 μM DCNP
(ii) 3.3 to 50 μM dopamine in the presence of 0.1, 0.67 or 3.3 μM DCNP
(iii) 3.3 to 133 μM DCNP in the presence of 0.1, 0.5 and 1 μM *p*-NP
(iv) 0.9 to 3 μM PAP^{35}S in the presence of 3.3 μM *p*-NP and at 0, 10, 50 and 100 μM DCNP.

Identification and characterization of DCNP-^{35}sulfate. DCNP-^{35}sulfate was generated using 67 μM DCNP, 0.3 μM PAP^{35}S, rat liver cytosol containing 200 μg protein and 50 mM KH_2PO_4–NaOH buffer at pH 8.0. The reaction mixture was incubated for 1 hr at 37°. DCNP was also generated in higher yield from 0.22 mM sodium ^{35}sulfate and 100 μM DCNP in the presence of 8 mM ATP–$MgCl_2$, rat liver cytosol containing about 300 μg protein and 50 mM KH_2PO_4–NaOH buffer, pH 8.0. This reaction mixture was incubated overnight at 37°. Both reactions were terminated by boiling for 1 min followed by centrifugation.

For the characterization of the products of hydrolysis of DCNP-^{35}sulfate, the latter was first isolated by HPLC using solvent system A (50 mM NaH_2PO_4, pH 6.2 containing 15% methanol) [15]. Acid hydrolysis of DCNP-^{35}sulfate biosynthesized from PAP^{35}S was carried out by boiling in 2 M HCl at pH 1 for 30 min. DCNP-^{35}sulfate, generated from sodium ^{35}sulfate, was subjected to both acid and enzymatic hydrolysis by 110 units of sulfatase at pH 5.0 for 90 min at 37°. Corresponding controls were included. Both the hydrolysates and controls were dried under a stream of nitrogen gas and resuspended in 100 μL of filtered water. The released products in the hydrolysates namely inorganic ^{35}sulfate and DCNP were identified and quantified by HPLC-radiometry and HPLC-DAD, respectively. The HPLC system consisted of an HP1090 (Hewlett–Packard) with an autosampler and a diode-array detector (HP1040A) connected in series to a Flo-one beta radioactive flow detector (Radiomatic Instrument and Chemical Co. Inc.). A solid cell was employed. The column (250 × 4.6 mm) was packed with Partisil ODS-3 of 10 μm particle size. The flow rate was 1 mL/min. For the UV detection of DCNP, the wavelength was set at 400 nm and quantification was carried out on an HP 3390 integrator.

Analysis of the labeled sulfate conjugates by HPLC-radiometry. Two different solvent systems were used for the analysis of the sulfates of DCNP, *p*-NP and dopamine. Solvent system B [17] which contained a mixture of 75 mM NaH_2PO_4, 0.35 mM sodium 1-octane sulphonic acid and 1 μM EDTA at pH 4.0, acetonitrile and methanol (85:4:11 by vol) was employed for the detection of DCNP- and *p*-NP-^{35}sulfates. Solvent system C comprises the same buffered solution as above containing 4% acetonitrile : methanol (98 : 2 v/v). This mobile phase was suitable for the separation of PAP^{35}S and the sulfate conjugates of DCNP and dopamine.

Analysis of harmol sulfate by HPLC-fluorimetry. The solvent system used was a mixture of methanol and 50 mM KH_2PO_4 with 2.5 mM sodium octylsulfate at pH 3.5 (50:50). The HPLC consisted of an HP1050 (Hewlett–Packard) with an autosampler, connected to a programmable fluorescence detector (HP1046A) and an integrator (HP3390). The flow rate was 0.3 mL/min. The settings on the detector were: excitation wavelength, 310 nm; emission wavelength, 420 nm; gain, 9. The column was 100 × 2.1 mm packed with Hypersil ODS of 5 μm particle size.

Protein determination. The protein content was determined by the Bradford method [18] using the Biorad reagent and bovine serum albumin as standard.

Table 1. Identification and quantification of the hydrolytic products of DCNP-^{35}sulfate by HPLC-radiometry and HPLC-DAD

Sample	Inorganic^{35}sulfate	DCNP-^{35}sulfate	DCNP
Control	—	1.7*	—
Acid hydrolysate	1.7	—	ND
Control	—	270, 307†	—
Acid hydrolysate	300	—	370
Enzymatic hydrolysate	33	267	ND

All values were expressed in pmol in 100 μL aliquot injected.
ND = not detectable by HPLC-DAD.
* Generated from 0.3 μM PAP^{35}S and 67 μM DCNP.
† Generated from 0.22 mM sodium^{35}sulfate and 100 μM DCNP.

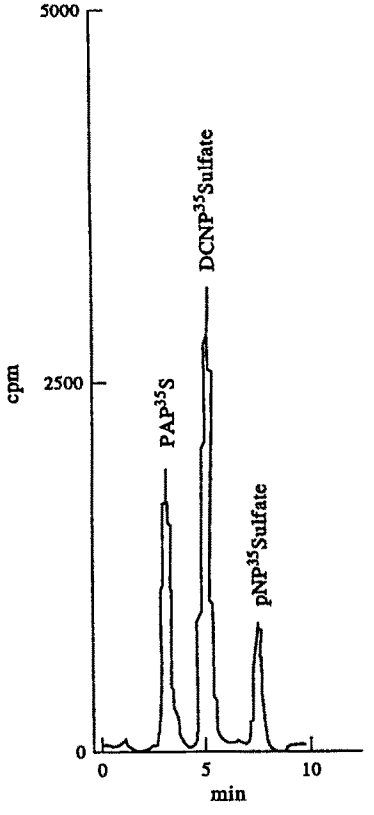

Fig. 1. HPLC chromatograph showing the separation of $PAP^{35}S$ (3.3 min), DCNP-^{35}sulfate (5.4 min) and *p*-NP-^{35}sulfate (7.7 min) using solvent system B.

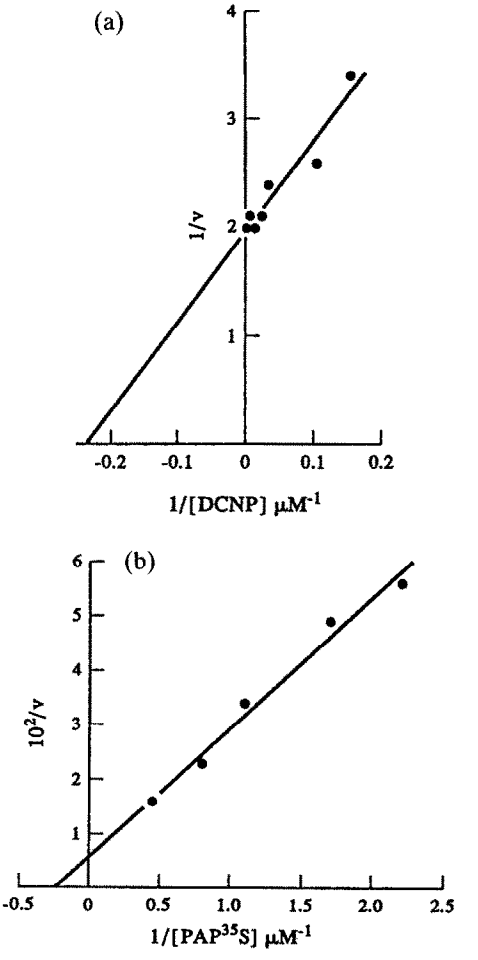

Fig. 2. Lineweaver–Burk plot of the formation of DCNP-^{35}sulfate by rat liver PST when (a) DCNP was varied from 6.7 to 500 μM. Substrate inhibition was observed at concentrations above 500 μM. (b) $PAP^{35}S$ was varied from 0.45 to 2.2 μM. The velocity, *v* was expressed as pmol DCNP-^{35}sulfate/min/mg protein.

RESULTS

Identification of DCNP-^{35}sulfate and characterization of its products of hydrolysis

DCNP was sulfated by rat liver PST *in vitro* and the sulfate conjugate was identified. With solvent system A, the retention times for $PAP^{35}S$/inorganic ^{35}sulfate, DCNP-^{35}sulfate and DCNP were 3.4, 5.7 and 17.0 min, respectively. The peak corresponding to DCNP-^{35}sulfate was not present in the control and was lost after acid hydrolysis of the reacted incubate. DCNP-^{35}sulfate, isolated by HPLC, on acid hydrolysis released inorganic ^{35}sulfate with a stoichiometry of almost 1:1 (Table 1); quantification was carried out by the HPLC–radiometric procedure developed for the separation of inorganic ^{35}sulfate from $PAP^{35}S$ [19]. However, the amount of DCNP released was not detectable by HPLC-DAD, a limitation imposed by the nanomolar concentration of $PAP^{35}S$ employed in the biosynthesis of DCNP-^{35}sulfate. In view of this, the sulfate conjugate was regenerated in higher amount from sodium ^{35}sulfate, which was introduced in micromolar concentration. Indeed, DCNP was detectable in the acid hydrolysate (Table 1) but digestion with sulfatase was minimal presumably due to its low activity in the commercial preparation of β-glucuronidase-sulfatase mixture.

Like other phenyl sulfate conjugates, the DCNP-^{35}sulfate formed was observed mainly in the supernatant when the double-precipitation procedure of Foldes and Meek [13] was followed. A small percentage, 10–20%, was precipitated when the reaction was carried out between pH of 8.5 and 9.5. Routinely in our assays, the PST reaction was terminated by boiling as some sulfate conjugates e.g. dopamine sulfate (to be discussed later) are partially precipitated by this work-up procedure.

Kinetics of single-substrate reactions

DCNP-^{35}sulfate, with retention time of 5.4 min was observed when DCNP was the substrate (Fig. 1). The DCNP–PST reaction exhibited classical Michaelis–Menten kinetics with a pH optimum of 8.0. The formation of DCNP-^{35}sulfate increased with increasing concentration of DCNP from 6.7 to 500 μM; substrate inhibition was apparent above 500 μM. A K_m of 4.3 μM was obtained (Fig. 2a) from the Lineweaver–Burk plot generated by the Enzpack software [20]. The velocity of reaction also

Table 2. Kinetic data for the sulfation, and inhibition of sulfation of *p*-NP, dopamine and DCNP

Varied substrate	K_m (μM)	Inhibitor	K_i (μM)	Type of inhibition
p-NP	0.2	DCNP	6.6	Competitive
Dopamine	9.2	DCNP	1.5	Competitive
DCNP	4.3	p-NP	0.044	Competitive
PAPS	4.0*	DCNP	57†	Noncompetitive

* DCNP as substrate.
† Measured with *p*-NP as substrate.

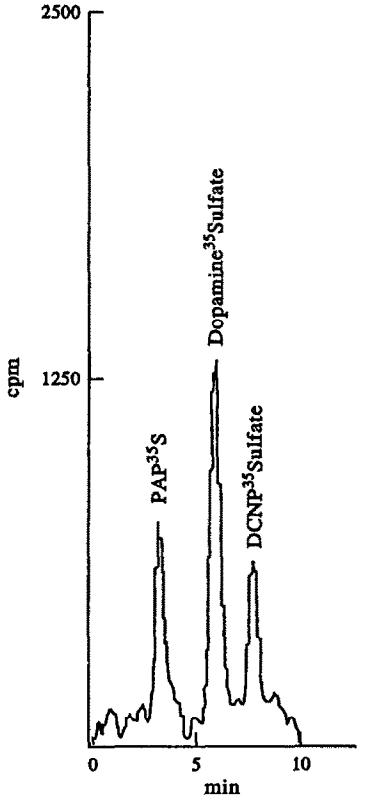

Fig. 3. HPLC chromatograph showing the separation of $PAP^{35}S$ (3.3 min), dopamine-^{35}sulfate (5.4 min) and DCNP-^{35}sulfate (6.8 min) using solvent system C.

increased with $PAP^{35}S$ concentration (0.45 to 2.2 μM) giving a K_m of 4 μM (Fig. 2b). The reaction proceeded linearly with enzyme concentration between 0 and 40 μg protein/incubate and with time of incubation up to 5 min. Michaelis–Menten kinetics were also established for the sulfation of *p*-NP and dopamine, with K_m values of 0.2 μM and 9.2 μM, respectively (Table 2).

Inhibition studies in mixed-substrate experiments

With DCNP as inhibitor. Two solvent systems (B and C) were shown to be suitable for the complete resolution of $PAP^{35}S$, DCNP-^{35}sulfate from *p*-NP-^{35}sulfate or from dopamine-^{35}sulfate (Figs 1 and 3). Thus each labeled sulfate conjugate could be quantified in the presence of the other in the two-substrate assays. With increasing concentration of *p*-NP, there was increased formation of *p*-NP-^{35}sulfate with a reciprocal decrease in DCNP-^{35}sulfate. Likewise, the reciprocal relationship was also observed by varying the DCNP concentration. DCNP exhibited mixed-competitive inhibition on the sulfation of *p*-NP (Fig. 4a) and of dopamine (Fig. 4b). However, when PAPS was the varied co-substrate, the sulfation of *p*-NP was inhibited non-competitively by DCNP (Fig. 5; Table 2).

With DCNP as substrate. The sulfation of DCNP was inhibited competitively by 0.1 to 1 μM *p*-NP (Fig. 6). However, higher concentrations of dopamine (of 0.5 to 100 mM) had to be introduced to produce inhibitory effects which were not dose-dependent (data not shown). From the sets of data which showed competitive inhibition (Figs 4 and 6), secondary plots were also generated, and the K_i values are shown in Table 2.

Comparison with the conventional double-precipitation procedure

The method of Foldes and Meek [13] was also used to measure *p*-NP-^{35}sulfate and dopamine-^{35}sulfate. Although the former conjugate was recovered almost quantitatively in the supernatant after double-precipitation by barium acetate, barium hydroxide and zinc sulfate, more than 50% of dopamine-^{35}sulfate was lost in the precipitate. This was evident on HPLC–radiometric analysis of a standard assay terminated by boiling as compared to precipitation by the conventional Foldes and Meek procedure.

Action of DCNP on the sulfation of p-NP, dopamine and harmol

DCNP between 10^{-7} and 10^{-3} M concentration showed differential inhibitory effects on the sulfation of *p*-NP, dopamine and harmol. At 10 μM DCNP, inhibition of sulfation of harmol, dopamine and *p*-NP was about 92, 78 and 15%, respectively (Fig. 7). Thus DCNP is a more powerful inhibitor of sulfation of harmol than that of *p*-NP and dopamine. This corroborates the findings of Mulder and Scholtens in their study of its comparative action on the sulfation of harmol and phenol [1].

DISCUSSION

Studies *in vivo* had shown the inhibitory effect of

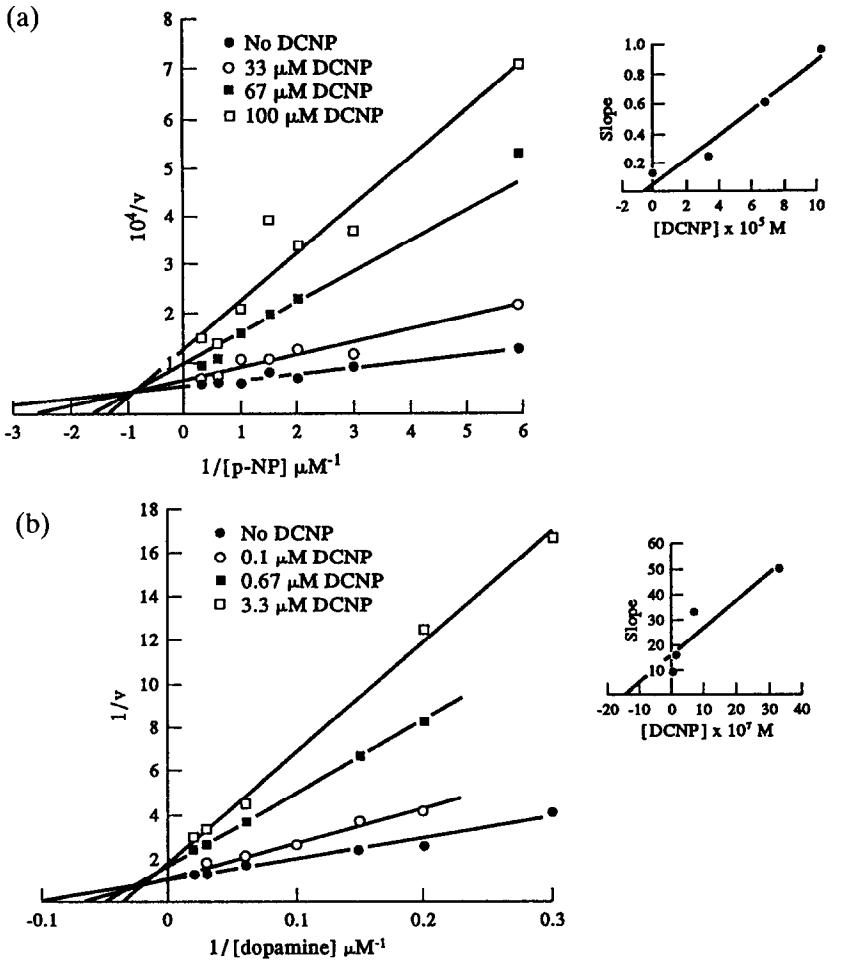

Fig. 4. Inhibition by DCNP on the sulfation of (a) *p*-NP and (b) dopamine. The velocity, *v* was expressed in cpm of *p*-NP-^{35}sulfate or pmol dopamine-^{35}sulfate/min/mg protein. Secondary plots of slope against concentration of DCNP are shown in the insets.

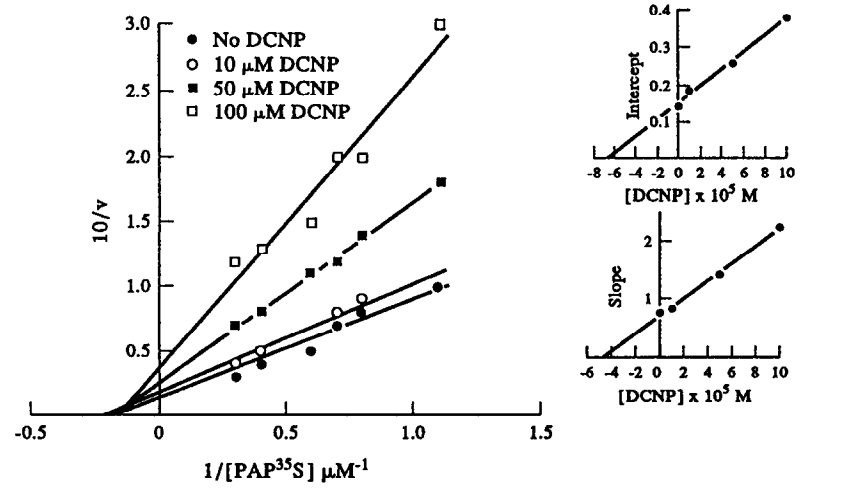

Fig. 5. Inhibition of sulfation of *p*-NP by DCNP when the concentration of PAP^{35}S was varied from 0.9 to 3 μM. The velocity, *v*, was expressed in pmol of *p*-NP-^{35}sulfate/min/mg protein. Secondary plots of intercept/slope against concentration of DCNP are shown in the insets.

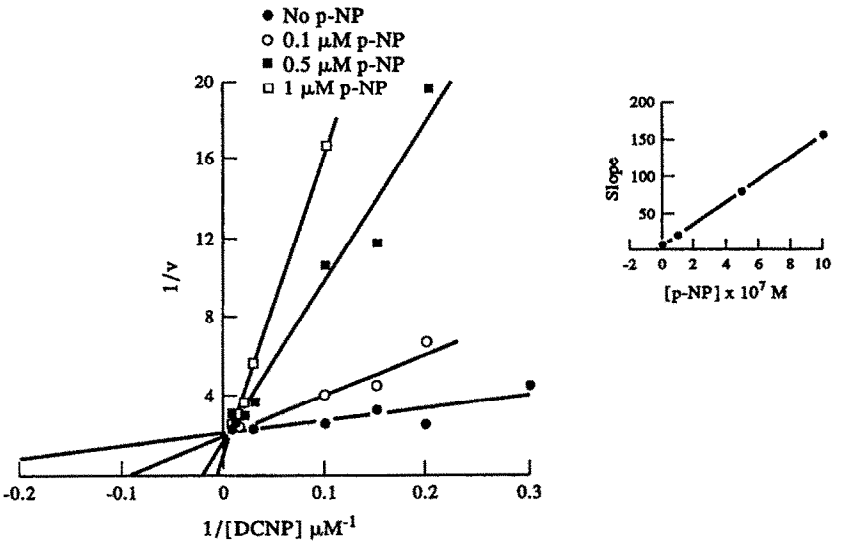

Fig. 6. Inhibition of sulfation of DCNP by *p*-NP. The velocity, *v* was expressed in pmol of DCNP-^{35}sulfate/min/mg protein. Secondary plot of slope against concentration of *p*-NP is shown in the inset.

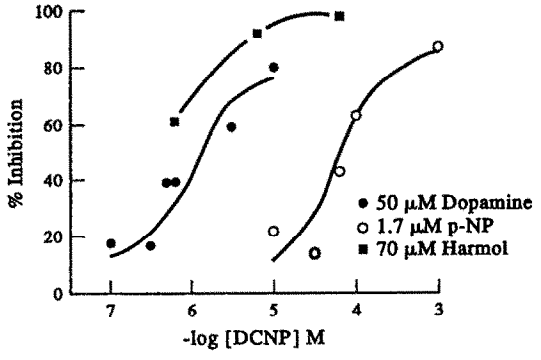

Fig. 7. Inhibition profile of rat liver PST by DCNP measured at 50 μM dopamine, 1.7 μM *p*-NP and 70 μM harmol. Results were compared to control values obtained in the absence of DCNP.

DCNP on sulfation to be immediate and long-lasting with no evidence of its metabolic transformation. This has led to the suggestion that it was probably absorbed rapidly, bound to proteins and eliminated slowly [21]. Its mechanism of action remains unknown although there was a suggestion that, like pentachlorophenol, it could be a dead end inhibitor [12, 15, 22] and its inhibition was reversible in studies *in vitro* and in liver perfusion [15, 21, 23–25]. In this paper we have demonstrated the sulfate conjugation of DCNP by a sulfotransferase present in rat liver cytosol and the reaction obeyed Michaelis–Menten kinetics. The identity of DCNP-^{35}sulfate was also confirmed by the release of inorganic ^{35}sulfate and DCNP in approximately stoichiometric ratio. DCNP as an inhibitor acted competitively in the sulfate conjugation of *p*-NP and dopamine, while as a substrate its own sulfation was likewise competitively inhibited by *p*-NP. However, when PAPS was the varied co-substrate, the sulfation of *p*-NP was inhibited non-competitively by DCNP. From the mechanism proposed by Spector and Cleland [26], the above kinetic data seemed to suggest that DCNP was an alternate-substrate inhibitor, with PAPS as the common co-substrate.

The classification of human PST into P and M forms was based on the selective inhibition by DCNP and this had been applied unambiguously to human PSTs as only two PST forms are present. Our data on the profile of inhibition at various concentrations of DCNP on the sulfation of *p*-NP and dopamine in rat liver cytosol differed considerably from those observed on human PST forms [3, 4] in that dopamine ST was more sensitive to inhibition by DCNP than *p*-NP-ST (Fig. 7). In the rat brain, the IC_{50} for DCNP was almost identical for these two substrates, showing little or no discrimination [15]. Thus the selective sensitivity of DCNP could not be generally applied to categorize PST isoforms in other species of animals where multiple PST forms may exist, as in the rat [10–12]. It was proposed that minoxidil sulfotransferase of rat liver has sequence similarity to human liver P-PST and could be the rat homologue of human P-PST [27]. Studies from our laboratory have also shown that both DCNP and *p*-NP inhibited minoxidil sulfotransferase in rat skin in a similar dose-dependent manner [28].

Under the conditions of the conventional double-precipitation method of Foldes and Meek [13], DCNP-^{35}sulfate was found to be water soluble. Therefore, whenever DCNP was used as an inhibitor, its sulfate conjugate could conceivably interfere with the measurement of the sulfation of *p*-NP or dopamine unless a separation step was introduced. The determination of dopamine sulfation by the established double-precipitation or modified single-

precipitation procedure was likely to be an underestimation as a considerable fraction (about 50%) of dopamine sulfate was precipitated. It was generally known that the recovery of the sulfate conjugates of acidic metabolites of the catecholamines was low, of the order of 25–42% [13, 29], but the phenyl sulfates of the parent amines were believed to be almost quantitatively recovered [13]. To overcome the uncertainty due to the differential solubility of the sulfated products, the conjugates formed were measured directly in this study using HPLC–radiometry. Chromatographic conditions were also developed for the measurement of PST activity with the model substrates *p*-NP and dopamine in the presence of DCNP, the classical inhibitor of the P form of human PST.

Acknowledgements—We thank the National University of Singapore for the research grant RP 87/349 and the research studentship awarded to Miss Valerie M.Y. Seah.

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