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Sulphate conjugation of minoxidil in rat skin

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Abstract—Minoxidil sulphotransferase (MST) activity was determined in the cytosolic fraction of rat skin and liver. MST of rat skin is similar to the P (phenol)-form of phenolsulphotransferase (PST) of human tissues with respect to thermostability and inhibition by 2,6-dichloro-4-nitrophenol (DCNP). *p*-Nitrophenol, a prototype substrate of human P-PST form, inhibits MST at micromolar concentration while millimolar concentrations of dopamine and tyramine, substrates of human M-(monoamine)-PST, are required to elicit a similar degree of inhibition. The enzymatic transfer of ^{35}S from sodium ^{35}Sulphate to minoxidil was also demonstrated suggesting that the rat skin is potentially capable of synthesizing 3′-phosphoadenosine-5′-phosphosulphate (PAPS) from inorganic sulphate and utilizing it for the biosynthesis of minoxidil sulphate, its active metabolite. Thus, it is conceivable that the pharmacological action of minoxidil as a promotor of hair growth could be carried out by the cutaneous tissues without the contribution of hepatic or other extrahepatic organs.

Materials and Methods

3′-Phosphoadenosine-5′-phospho-^{35}Sulphate ($PAP^{35}S$*) of specific radioactivity 2.5 Ci/mmol (concentration 0.448 μmol/mL) and sodium ^{35}Sulphate with specific radioactivity of 368.05 mCi/mmol were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). Minoxidil was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Preparation of cytosolic extract of rat skin and liver. Hair from the back of the rat was shaven with a sharp razor and a small area of about 5 cm square was excised. After removal of the subcutaneous tissue, the whole skin was cut into pieces with scissors and a 40% homogenate was prepared in 0.15 M KCl containing 3 mM dithiothreitol (DTT) using the Polytron homogenizer (Kinematica GmbH, Luzern, Switzerland). This was rehomogenized with a motorized teflon pestle. The supernatant obtained after centrifugation at 105,000 *g* was used in the assay of minoxidil sulphotransferase (MST) and for the overall sulphate conjugation of minoxidil from inorganic ^{35}Sulphate and ATP. The cytosolic extract of rat liver was prepared as described previously [1].

Measurement of MST activity. Ethanol present in the commercial preparation of $PAP^{35}S$ was removed by evaporating it under a stream of oxygen-free nitrogen gas. Routinely a volume of 10 μL $PAP^{35}S$, representing a final concentration of 0.6 μM was used in the MST assay. Suboptimal concentrations of $PAP^{35}S$ are invariably used in the measurement of phenolsulphotransferase (PST) activity. To this was added minoxidil (2.1 mM), 50 mM KH_2PO_4-NaOH buffer of pH 7.0 and 35 μL skin or rat liver cytosolic fraction containing between 0.2–0.3 mg and 0.1–0.3 mg protein, respectively. The total final volume of the reaction incubate was 150 μL. Incubation was carried out at 37° in a water bath and the reaction was terminated by adding 1 mL ethyl acetate and 0.4 mL of 1 M ammonium hydroxide. The incubation time was 15 and 5 min, respectively, for the measurement of MST activity in skin and liver extracts. Minoxidil ^{35}Sulphate present in 0.2 mL of the organic phase was quantified by counting the radioactivity in 5 mL Universal scintillation fluid, following the procedure of Johnson and Baker [2]. The specific activity of MST was determined by extrapolating from a standard curve of $PAP^{35}S$ measured at the same time. Controls were carried out by omitting minoxidil in the assay mixture.

The overall sulphate conjugation of minoxidil. The assay incubate contained the following reagents: sodium ^{35}Sulphate (13.6 μM), 20 μL each of 140 mM ATP and Mg^{2+} in 30 mM DTT (5.6 mM), 100 μL of 8 mM minoxidil (1.6 mM) and 50 mM glycine-NaOH buffer at pH 8.6 in a total volume of 500 μL. Values in parentheses represent final concentrations of reactants. The reaction was started by introducing 50 μL of the rat skin supernatant containing about 0.2 mg protein. After incubation for 10–120 min, the amount of minoxidil ^{35}Sulphate was determined as described and quantified by extrapolating from a standard curve of sodium ^{35}Sulphate.

Effect of 2,6-dichloro-4-nitrophenol (DCNP) and thermostability test on MST. DCNP, a selective inhibitor of the P form of human PST [3] was added at a final concentration of 10^{-9} to 10^{-4} M to the standard MST assay. The protocol for the thermostability test of Reiter and Weinshilboum [4] was followed: the skin cytosolic fraction was pre-incubated at 43° for 15 min in a water bath. The MST activity was compared to the same cytosolic fraction that was not subjected to this thermal effect.

Action of P and M substrates on MST activity. Representative substrates of the P and M forms of human PST, namely *p*-nitrophenol and dopamine or tyramine, respectively, were added at 10^{-8} to 10^{-3} M concentration to the MST assay. All measurements were carried out at 1 mM concentration of minoxidil.

Protein determination. This was carried out by the method of Bradford [5] using the Bio-Rad dye procedure with bovine serum albumin as standard.

Results and Discussion

Kinetic data of MST activity in rat skin and liver extract. MST has a pH optimum between 7 and 7.4 and the rate of reaction was linear for 15 and 5 min, for the enzyme extracted from rat skin and liver, respectively. Under the assay conditions described, the overall sulphate conjugation of minoxidil in rat liver extract increased progressively up to 1 hr. From the Lineweaver–Burk [6] plots generated by the Enzpack program [7], the apparent K_m values for PAPS and minoxidil for the MST reaction are shown in Table 1 and Figs 1 and 2. The rates of formation of minoxidil ^{35}Sulphate in rat skin cytosol from $PAP^{35}S$ and from sodium ^{35}Sulphate and ATP were, respectively, 4.2 and 5.6 pmol minoxidil ^{35}Sulphate/min/mg protein. The corresponding values, expressed in the same units, were 35 and 222 for rat liver MST. It must be noted that the two sets of data, representing MST activity and the overall sulphate conjugation of minoxidil, cannot be compared directly as (a) the former is a one-step MST reaction while the latter is a measurement of three enzymatic reactions catalysed

* Abbreviations: PAPS, 3′-phosphoadenosine-5′-phosphosulphate; PST, phenolsulphotransferase; MST, minoxidil sulphotransferase; DCNP, 2,6-dichloro-4-nitrophenol.

Table 1. Sulphate conjugation of minoxidil by rat skin and liver cytosolic fractions measured using PAP^{35}S in the MST assay and sodium ^{35}sulphate in the overall sulphate conjugation reaction

	Rat skin	Rat liver
K_m for PAPS (μM)	0.6	5
V_{max}*	6.0	472
K_m for minoxidil (mM)	0.3	0.2
V_{max}*†	4.0	68
Specific activity* measured at 0.6 μM PAP^{35}S	4.2	35
Overall sulphation* measured at 13.6 μM sodium ^{35}sulphate	5.6	222

For details of reaction conditions, see Figs 1 and 2.
* In pmol minoxidil ^{35}sulphate/min/mg protein.
† These values were obtained under sub-optimal concentrations of PAP^{35}S.

by ATP sulphurylase, APS kinase and MST, and (b) the concentrations of sodium ^{35}sulphate and PAP^{35}S employed differed considerably. From the kinetic measurements, it was observed that the K_m values for minoxidil were similar in rat skin and liver but those for PAPS varied by one order of magnitude (Table 1, Figs 1 and 2). The higher apparent K_m of 5 μM for PAPS for rat liver MST was also obtained when MST measurements were made employing a wider range of PAP^{35}S from 0.01 to 9 μM. Whether the difference in binding affinity for the "active sulphate" is due to different isoforms of MST in the two tissues merits further study. The capacity for sulphate conjugation was relatively low in the skin as reflected in the V_{max} values (see Table 1). There is probably a quantitative difference in MST and PAPS-generating enzymes in the two tissues. Preliminary studies have shown that PAPS generation in the skin extract could be measured by a method modified from that reported previously [8] from this laboratory (unpublished observation).

Effect of heat treatment and DCNP, p-nitrophenol, dopamine and tyramine on MST activity. When the rat skin cytosolic extract was subjected to incubation at 43° for 15 min, about 50% of the MST activity was retained, showing that it is relatively thermostable. Under the same

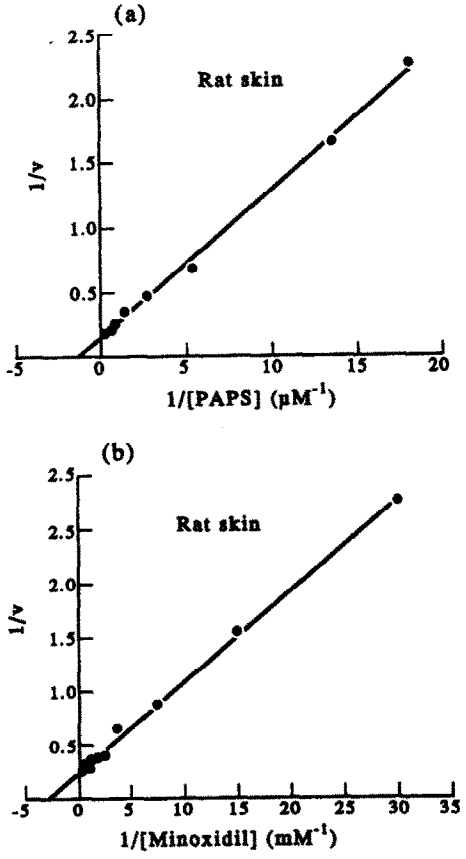

Fig. 1. Lineweaver–Burk plots of MST activity in rat skin where velocity (v) is expressed as pmol minoxidil ^{35}sulphate/min/mg protein against: (a) 0.06–3.67 μM PAP^{35}S measured at 1.1 mM minoxidil; (b) 0.03–2.1 mM minoxidil measured at 0.6 μM PAP^{35}S.

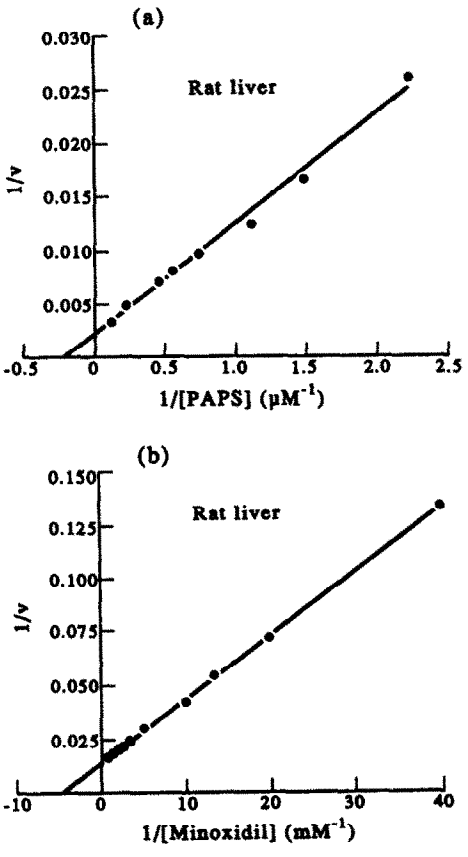

Fig. 2. Lineweaver–Burk plots of MST activity in rat liver where velocity (v) is expressed as pmol minoxidil ^{35}sulphate/min/mg protein against: (a) 0.5–9.0 μM PAP^{35}S measured at 1.6 mM minoxidil; (b) 0.025–1.6 mM minoxidil measured at 0.5 μM PAP^{35}S.

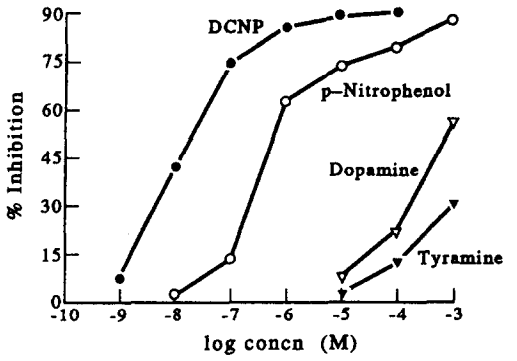

Fig. 3. Inhibition of MST activity in rat skin cytosol by DCNP, *p*-nitrophenol, dopamine and tyramine.

condition of heat treatment, the thermolabile or M form of human PST would be 95% inactivated [4]. DCNP and *p*-nitrophenol were shown to inhibit MST activity of rat skin cytosol in a dose-dependent manner from 10^{-9} to 10^{-3} M (Fig. 3). Inhibition was only observed at higher concentrations (10^{-5} to 10^{-3} M) of dopamine and tyramine; these biogenic amines are substrates of human M-PST [3]. The data presented above suggest that rat skin MST is more like the P form of human PST. This concurs with the reports on MST measured in human platelets [2], rat and human liver [9, 10].

In relation to hair growth, minoxidil sulphate was identified to be the active metabolite [11]. The enzyme involved, MST has been located in the outer root sheath of the hair follicle [12] and its activity was reported to be induced during the differentiation of epithelial keratinocytes [13]. This paper addresses the localization of MST in rat skin and the enzymatic transfer of ^{35}sulphate from sodium ^{35}sulphate to minoxidil in rat skin. These observations suggest that the skin is potentially capable of synthesizing PAPS from inorganic sulphate and employing it for the biosynthesis of minoxidil sulphate. This could perhaps explain the efficacy of minoxidil when applied topically. The identification of MST extends the list of enzymes involved in detoxication in skin cells [14]. It would be interesting to trace the fate of the sulphate moiety of minoxidil sulphate to hair proteins. In this context, its transfer to synthetic peptides non-enzymatically [15] and to endogenous proteins of smooth muscle [16] has been reported. Minoxidil sulphate shares with other drugs of dissimilar structures the ability to open potassium channels and this has been proposed to be its mechanism of action in hair growth [17].

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REFERENCES

1. Khoo BY, Sit KH and Wong KP, Sulphate conjugation in human liver: direct measurement of *N*-acetyldopamine sulphate. *Biochem Pharmacol* **40**: 1021–1026, 1990.
2. Johnson GA and Baker CA, Sulfation of minoxidil by human platelet sulfotransferase. *Clin Chim Acta* **169**: 217–228, 1987.
3. Rein G, Glover V and Sandler M, Multiple forms of phenolsulfotransferase in human tissues. Selective inhibition by dichloronitrophenol. *Biochem Pharmacol* **31**: 1893–1897, 1982.
4. Reiter C and Weinshilboum RM, Acetaminophen and phenol: substrates for both a thermostable and thermolabile form of human platelet phenol sulfotransferase. *J Pharmacol Exp Ther* **221**: 43–51, 1982.
5. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
6. Lineweaver H and Burk D, The determination of enzyme dissociation constants. *J Am Chem Soc* **56**: 658–666, 1934.
7. Williams PA, Enzpack, Version 2.0, Elsevier Science, The Netherlands, 1985.
8. Wong KP, Khoo BY and Sit KH, Biosynthesis of PAPS *in vitro* by human liver. Measurement by two independent assay procedures. *Biochem Pharmacol* **41**: 63–69, 1991.
9. Hirshey SJ and Falany CN, Purification and characterization of rat liver minoxidil sulphotransferase. *Biochem J* **270**: 721–728, 1990.
10. Falany CN and Kerl EA, Sulfation of minoxidil by human liver phenol sulfotransferase. *Biochem Pharmacol* **40**: 1027–1032, 1990.
11. Buhl AE, Waldon DJ, Baker CA and Johnson GA, Minoxidil sulfate is the active metabolite that stimulates hair follicles. *J Invest Dermatol* **95**: 553–557, 1990.
12. Dooley TP, Walker CJ, Hirshey SJ, Falany CN and Diani AR, Localization of minoxidil sulfotransferase in rat liver and the outer root sheath of anagen pelage and vibrissa follicles. *J Invest Dermatol* **96**: 65–70, 1991.
13. Johnson GA, Baker CA and Knight KA, Minoxidil sulfotransferase, a marker of human keratinocyte differentiation. *J Invest Dermatol* **98**: 730–733, 1992.
14. Pham M-A, Magdalou J, Siest G, Lenoir M-C, Bernard BA, Jamoulle J-C and Shroot B, Reconstituted epidermis: a novel model for the study of drug metabolism in human epidermis. *J Invest Dermatol* **94**: 749–752, 1990.
15. Groppi VE, Burnett B-A, Maggoria L, Bannow C, Zurcher-Neeley HA, Heinrikson RL, Schostarez HJ and Bienkowski MJ, Protein sulfation: a unique mechanism of action of minoxidil sulfate. *J Invest Dermatol* **94**: 532, 1990.
16. Meisheri KD, Oleynek JJ and Puddington L, Role of protein sulfation in vasodilation induced by minoxidil sulfate, a K^+ channel opener. *J Pharmacol Exp Ther* **258**: 1091–1097, 1991.
17. Buhl AE, Waldon DJ, Conrad SJ, Mulholland MJ, Shull KL, Kubicek MF, Johnson GA, Brunden MN, Stefanski KJ, Stehle RG, Gadwood RC, Kamdar BV, Thomasco LM, Schostarez HJ, Schwartz TM and Diani AR, Potassium channel conductance: a mechanism affecting hair growth both *in vitro* and *in vivo*. *J Invest Dermatol* **98**: 315–319, 1992.