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S-METHYLATION OF CAPTOPRIL

DEMONSTRATION OF CAPTOPRIL THIOL METHYLTRANSFERASE ACTIVITY IN HUMAN ERYTHROCYTES AND ENZYME DISTRIBUTION IN RAT TISSUES

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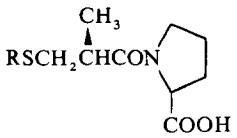
Abstract—The presence of a methyltransferase enzyme in human red blood cells (RBCs) capable of *S*-methylation of captopril is described. The apparent Michaelis–Menten (K_m) constant for captopril was 0.5 mM and the maximum velocity (V_{max}) was 0.391 pmoles *S*-methylcaptopril (mg protein)$^{-1}$ min^{-1}. There is some evidence presented to show that *S*-methylcaptopril inhibited its own formation with a k_i value of 5.81 mM. Captopril thiol methyltransferase activity was also examined in rat tissues and was found to be present in all tissue studied. Subcellular localisation studies in rat liver suggest that the enzyme was microsomal in origin. The order of activity was liver > heart > spleen > lung > kidney ⩾ RBC (rat). This tissue distribution was quite different from previous studies using other thiol substrates and is consistent with more than one form of thiol methyltransferase enzyme in tissues.

Captopril (CP)† is a novel aliphatic sulfhydryl drug that is becoming increasingly used for the treatment of hypertension and congestive heart failure [1]. Side effects reported from use of this drug include rashes, taste loss, neutropenia and occasionally proteinuria and it has been speculated that these adverse effects could be due to the reactive free thiol group [1]. Although the metabolism of CP has not been well defined there have been recent reports of an *S*-methyl metabolite of CP in human [2] and rat [3] urine. Moreover, urinary excretion of SMC in man was only evident after chronic dosing and not following a single dose of CP [2].

A soluble thiol methyltransferase enzyme has been reported in human RBC lysates capable of *S*-methylation of 6 MP and 2-thiouracil [4]. *S*-Methylation has also been reported to occur for aliphatic thiols by a membrane-bound thiol methyltransferase enzyme in human RBC [5] and in liver microsomal preparations [6, 7].

This paper describes the ability of thiol methyltransferase enzymes in human RBC lysates to *S*-methylate CP and then reports on the tissue distribution of this enzyme activity in the rat.



R = H, CP; R = CH_3, SMC.

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† Abbreviations: CMT, captopril thiol methyltransferase; CP, captopril; SMC, *S*-methylcaptopril; RBC, red blood cell; SAM, *S*-adenosyl-L-methionine; 6MP, 6-mercaptopurine; NEM, *N*-ethylmaleimide.

METHODS

Preparation of human RBC lysates. Blood from healthy human volunteers was collected by venepuncture into heparin-tubes and centrifuged at 2000 *g* for 10 min. Plasma was removed and the RBCs were resuspended in an equal volume of normal saline and recentrifuged. This washing procedure was repeated twice. The washed RBCs were lysed in 5 vol. of ice-cold water and centrifuged at 10,000 g_{max} for 10 min. The supernatant was used fresh for the determination of enzyme activity, or aliquots were frozen in liquid nitrogen and stored at −20°.

Preparation of tissue supernatants. Normal healthy Sprague–Dawley rats of either sex (weight 160–200 g) were killed by cervical dislocation. Tissues including heart, lung, spleen, liver and kidney were freshly excised and washed free of blood with ice-cold normal saline. Blood was also studied and treated as for human RBC lysates. Tissues (except for blood) were finely diced and homogenized in 10 vol. of potassium phosphate buffer, 10 mM, pH 7.5, with an Ultra-Turrax (TP18-10) for 15 sec, and finally with a Dounce homogenizer (10 strokes). Homogenates were then filtered through cheese cloth and centrifuged at 10,000 g_{max} for 10 min. The supernatant was used for measurement of enzyme activity.

Preparation of subcellular fractions. Rat liver homogenates were subject to differential centrifugation in 0.25 M sucrose. The cellular membranes and nuclei were obtained from a 1000-g_{max}, 10-min pellet. The supernatant from this fraction was then subjected to a 10,000-g_{max}, 10-min centrifugation and finally the supernatant from this fraction was then subjected to a 100,000-g_{max}, 60-min centrifugation. All pellets were resuspended in 0.25 M sucrose and aliquots of these and the final supernatant were assayed for CMT activity.

Measurement of enzyme activity (CMT assay). One hundred microlitre aliquots of RBC lysates or tissue supernatant fractions were added to 10-ml stoppered glass conical centrifuge tubes. Twenty-five microlitres of a potassium phosphate buffer, 150 mM, pH 7.5, and 5 μl of 120 mM CP (in dimethylsulphoxide) were added (final concentration 4 mM). The reactions was initiated by the addition of 25 μl of the following reagents (final concentration in tube): non-radioactive SAM 25 μM, *S*-adenosyl-L-[methyl-^{3}H]methionine 30 KBq, dithioerythritol 1 mM in potassium phosphate buffer. The mixture was incubated in a shaking water bath at 37° for 30 min; and the reaction was stopped by the addition of 100 μl of 2 M hydrochloric acid. Five millilitres of ethyl acetate was added, the tubes were stoppered and were mixed vigorously on a vortex mixer for 15 sec. Following centrifugation at 1000 *g* for 2 min, 4 ml of the organic phase was removed and placed into plastic liquid scintillation vials. The ethyl acetate was evaporated to dryness under a gentle stream of air and 10 ml of a toluene fluor {2,5-diphenyloxazole [0.4% (w/v)] and 1,4-di-2,5-phenyloxazolylbenzene [0.01 (w/v) in toluene]} added. Radioactivity was determined in a Packard 3380 liquid scintillation counter.

Blank samples included all reagents except CP substrate. CMT activity was expressed as pmoles of SMC formed (mg protein)$^{-1}$ min^{-1} of incubation at 37°. All results were corrected for counting efficiency and for the extraction efficiency of SMC which was 75%.

Exactly the same procedure was used when 6-MP (in dimethylsulphoxide) was used as the substrate (0.03–4 mM), except that the reaction was stopped by the addition of 0.5 ml of borate buffer, 0.5 M, pH 10, and the radioactivity was extracted out with 5 ml of 20% (v/v) isoamyl alcohol in toluene as described by Weinshilboum *et al.* [4]. Ten millilitres of toluene fluor was added directly to these extracts and counted for radioactivity.

TLC. Reaction tubes following the incubation were treated with 1 mg NEM (100 μl of 10 mg/ml solution in water) for 15 min to alkylate the free sulfhydryl group. These contents were then acidified with 100 μl of 2 M hydrochloric acid and extracted with ethyl acetate. The dry extract was evaporated and reconstituted with 50 μl of methanol.

The radioactive products were identified by TLC on silica gel sheets 200 μM thick (Merck, Darmstadt, F.R.G.). The solvent system used was benzene/acetic acid (3:1).

After development SMC spots were identified either by a chloroplatinic acid spray or segments of the chromatograms were placed in counting vials that contained 1.0 ml of Soluene-100 (Packard Instrument Co. Inc.). After 30 min, vials were counted for tritium radioactivity using a toluene-based cocktail. Quench of samples was corrected by automatic external standardisation.

Protein determinations. The Lowry method [8] was used to measure protein concentrations in erythrocyte lysate and tissue supernatants. Bovine serum albumin was used as a standard for the protein assay.

Estimation of K_m and V_{max} using SCAFIT. The kinetic parameters K_m and V_{max} were evaluated by a computer program developed by Munson and Rodbard [9] called SCAFIT which was part of a larger package known as LIGAND. This is a weighted, non-linear curve-fitting program which provides a more accurate estimate of the kinetic parameters than conventional Lineweaver–Burk plots since points most subject to error (low substrate concentration) are not as heavily weighted by the non-linear analysis as they are by graphical means.

MATERIALS/REAGENTS

S-Adenosyl-L-[methyl-^{3}H]methionine (sp. act. 2.22 TBq/mmole) was purchased from Amersham International Ltd (Amersham, U.K.). Commercial supplies of SAM hydrogen sulfate (Boehringer-Ingelheim, Darmstadt, F.R.G.), 6MP (Fluka, Buchs, Switzerland), dithioerythritol (Calbiochem, San Diego, CA) and NEM (Sigma), were used. CP and CP disulfide dimer were a generous gift from Squibb Pharmaceuticals (Princeton, NJ), SMC was synthesized as described previously and verified by gas chromatography–mass spectrometry [10]. Chloroplatinate spray reagent was prepared from 10 ml of 0.002 M chloroplatinic acid, 0.6 ml of 1 M potassium iodide, 1 ml of 6 M hydrochloric acid and 50 ml of acetone (commercial grade).

RESULTS

Selection of assay conditions

The selection of the appropriate assay conditions for measurement of CMT activity was based primarily on those described by Weinshilboum *et al.* [4] for 6MP thiol methylation in human RBC lysates. However, both allopurinol and depletion of Mg^{2+} by a Chelex-100 resin were omitted from the procedure since CP was not likely to be a substrate for either of the enzymes known to degrade purines. In addition, there did not appear to be any difference in enzyme activity in the presence or absence of Mg^{2+} using CP as substrate, suggesting that the enzyme was not a Mg^{2+}-dependent transferase.

A 'no-substrate' blank was routinely used for measurement of 'blank' radioactivity in samples mainly resulting from formation of radioactivity labelled methanol from SAM [12]. Both 'buffer blanks' (tubes with buffer instead of enzyme) and heated tissue blanks (tubes with enzyme previously heated to 80° for 5 min) gave consistently lower activities (*ca.* 5000 dpm) than were present in 'no substrate' blanks (25,000 dpm). The dpm in active samples were of the order of 50,000 dpm.

Effect of protein and time on CMT activity

CMT activity was linear with time up to 90 min [Fig. 1(A)]. Assays for human RBC lysates were usually incubated for 60 min whereas tissue supernatants were incubated for 30 min. CMT activity increased in a linear fashion with increasing human RBC lysate protein up to 8.2 mg/tube [Fig. 1(B)]. This amount was equivalent to about 20 μl of packed RBC. Typically half this amount was present in the incubation tubes.

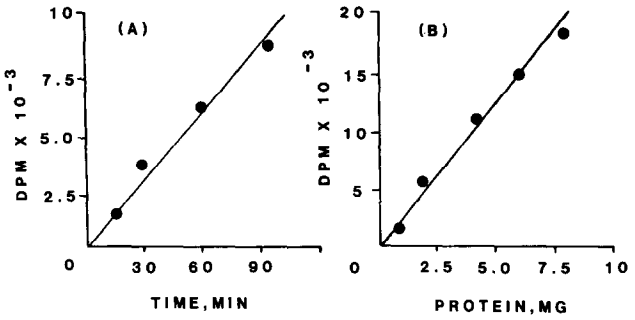

Fig. 1. Effect of: (A) increasing time of incubation, and (B) increasing quantities of human erythrocyte lysate on captopril thiol methyltransferase activity.

Effect of enzyme activity on substrate concentration

The enzyme activity of human RBC lysates CMT was measured in the presence of varying concentrations of CP and in the presence of a saturating amount of SAM (25 μM) (Fig. 2). The K_m for SAM was 3 μM. Enzyme activity in these experiments was expressed as a percentage of the maximum attainable increase to facilitate comparison from one experiment to another since absolute enzyme activities varied from one batch to another.

There was a concentration-dependent increase in enzyme activity in the CP concentration range 0.12–4 mM. However, when the concentration of CP in the incubation medium exceeded 4 mM there was a reduction in enzyme activity compared to that achieved at 2 and 4 mM. At 32 mM the CMT enzyme activity was reduced to 23% of the maximum (Fig. 3). The calculated Michaelis–Menten constant (K_m) from a total of six separate experiments was 0.5 mM (S.E. = 0.1). The V_{max} using SCAFIT was 0.391 pmoles SMC (mg protein)$^{-1}$ min^{-1} (S.E. = 0.007).

TLC of reaction product

TLC of the ethyl acetate extract from an assay of CMT following reaction with NEM gave rise to one major single peak with an R_f of 0.53 which coincided with that of authentic SMC. CP and other related analogues were all separated in this system from SMC. Both unreacted captopril (as NEM adduct) and the oxidized form CP disulfide dimer had R_f values of 0.34 and 0.20. Other known CP oxidation products had R_f values less than 0.2. The addition of NEM was necessary since free CP co-elutes with SMC in this system, and prevents further oxidation of CP to the dimer. There was usually a small spot of radioactivity (5% of SMC) at $R_f = 0.20$ which appeared in the reaction products coinciding with the disulfide dimer. This is probably some dimer formed as a result of spontaneous oxidation of CP in the incubation medium and exchange of labile tritium to 'active-hydrogen' molecules in the CP molecule.

Inhibitors of RBC CMT activity

The decrease in CMT activity observed with substrate concentrations in excess of 4 mM was investigated as a probable inhibition by the product of the reaction, SMC. In a series of experiments SMC at concentrations of 1, 4 and 8 mM was included (in quadruplicate) in reaction mixtures containing 4 mM CP and human RBC lysates. The results show a 31%

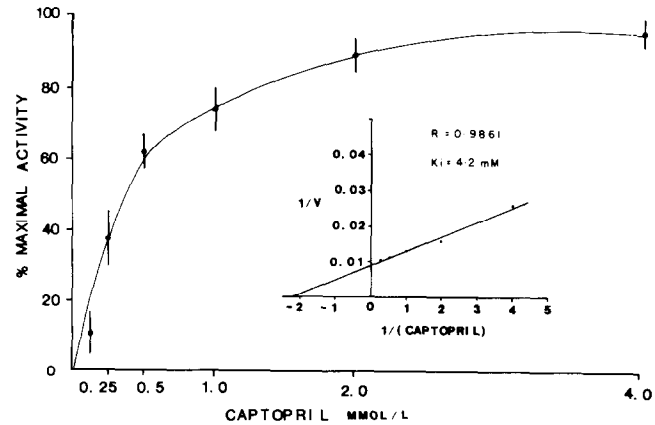

Fig. 2. Increase in captopril thiol methyltransferase activity as a function of captopril concentration using a human erythrocyte lysate enzyme preparation. Results are expressed as means ± S.E.M. (N = 6). Insert: Lineweaver–Burk plot of reciprocal velocity against the reciprocal concentration of captopril. The K_m obtained from this plot was 0.471 mM. The least-squares regression line is shown ($r = 0.993$, $P < 0.001$).

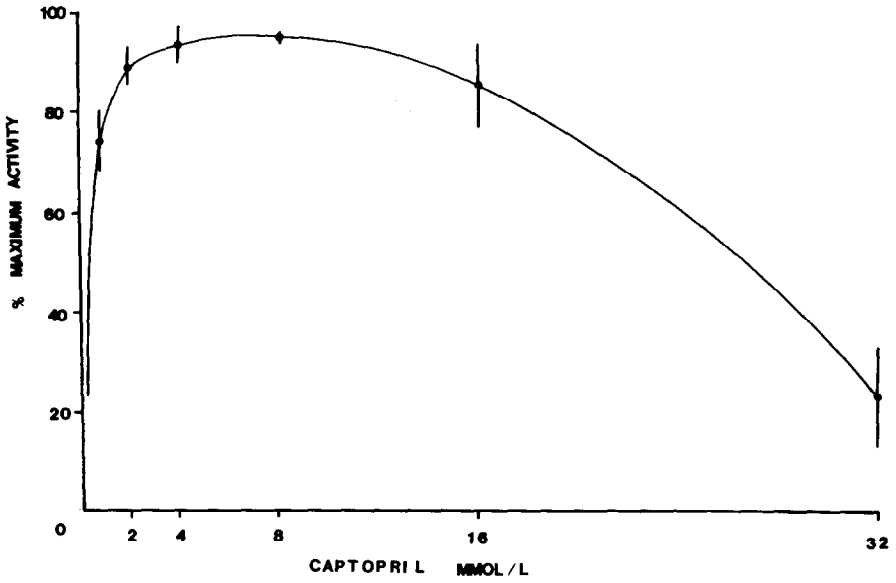

Fig. 3. Effect of captopril thiol methyltransferase enzyme activity as a function of captopril concentration (mM). Results are expressed as means of the percentage increase in enzyme activity ± S.E.M. (N = 4).

inhibition of the enzyme ($P < 0.02$) at a concentration of 1 mM which increased to a 62% inhibition at 8 mM ($P < 0.01$). A Dixon plot ($1/Vs\,[I]$) (Fig. 4) shows a linear relationship with these data giving an apparent K_i of 5.81 mM. Another known metabolite of CP metabolism, the disulfide dimer of CP, did not appear to inhibit formation of SMC from CP at concentrations as high as 8 mM.

Effect of storage on enzyme activity

Frozen RBC lysate prepared from human blood demonstrated a 2-fold loss in activity when stored at −20° over a period of 3 weeks. During the first week of storage the activity of CMT was 0.36 pmoles (mg protein)$^{-1}$ min^{-1}. This had declined to 0.24 units in the second week and 0.17 units in the third week. Use of enzyme preparations within a week of storage would appear to be mandatory. Experiments in rats were always performed with tissue supernatants prepared on the same day.

Comparison with 6MP as substrate

The use of 6MP as the substrate for thiol methyltransferase enzymes in human RBC lysates has previously been shown [4]. We performed a series of experiments to verify the viability of our enzyme preparation in this lysate preparation.

There was a concentration-dependent increase in enzyme activity with this substrate in the concentration range 0.03–1 mM with a K_m of 0.1 mM and a V_{max} of 0.56 pmoles 6MP (mg protein)$^{-1}$ min^{-1}. The V_{max} for 6MP as substrate was slightly higher than when CP was substrate. The K_m for 6MP was 3-fold lower than reported by Weinshilboum *et al.* [4]

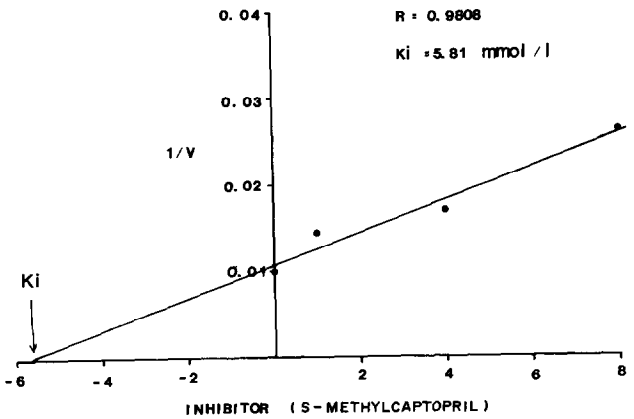

Fig. 4. Dixon plot of reciprocal velocity against inhibitor (*S*-methylcaptopril) concentration (mM) for inhibition of captopril thiol methyltransferase activity in human erythrocyte lysates. The K_i obtained from this plot was 5.81 mM. The least-squares regression line is shown ($r = 0.9808$, $P < 0.05$).

despite the fact that 6MP-degrading enzymes in the lysate were not inactivated as previously described [4].

Rat tissue distribution of CMT

The activity of CMT in rat liver, kidney, spleen, lung and heart post-mitochondrial fractions (10,000 g_{max} for 10 min) was examined in four or five separate experiments. Activity was generally high with the greatest activity present in liver [26 pmoles SMC (mg protein)$^{-1}$ min^{-1}] which was some 72 times the activity observed in human RBC lysates [$P < 0.05$ (unpaired t-test)] (Table 1). Other rat tissues examined, including heart, spleen and lung, showed relatively high CMT activities, which were not significantly different from that of liver ($P > 0.05$). Kidney exhibited significantly less activity than liver ($P < 0.05$) but was greater than human RBC lysates [10.8-fold ($P < 0.05$)] and rat RBC lysates [65-fold ($P < 0.05$)]. In contrast to human RBC lysates, rat RBC lysates showed barely detectable CMT activity (Table 1).

Subcellular localization of CMT

Subcellular localization of CMT (N = 3) showed that the microsomal fraction (100,000-g_{max}, 60-min pellet) contained the majority of CMT activity (being 51 ± 11% of the total recovered enzyme activity [11]), whilst the particulate fractions containing mitochondria (10,000-g_{max}, 10-min) and nuclei and cell membranes (1000-g_{max}, 10-min) gave 42 ± 10 and 3 ± 3% of the total recovered enzyme activity. The soluble fraction contained 4 ± 4% of the total activity. The sp. act. of the microsomal fraction was also highest at 21.6 ± 1.6 pmoles SMC min^{-1} (mg protein), which was no different from the crude 10,000-g_{max}, 10-min liver supernatant used in earlier structures [$P > 0.05$ (unpaired t-test)].

DISCUSSION

CP is a unique sulfhydryl-containing inhibitor of angiotensin-converting enzyme. Its use as an antihypertensive agent has been restricted, largely due to the occurrence of unwanted side effects [1]. It is thought that the reactive free thiol group plays a key role in these adverse reactions. Knowledge about its metabolism, particularly metabolism of the sulfur, is therefore necessary to fully understand its pharmacological actions. Recently, an *S*-methyl metabolite of CP has been described in urine of patients receiving CP. This metabolite was only present after chronic use of the drug [2]. This observation led us to believe that thiol methyltransferase enzymes play a role in the metabolism of CP and that the activity of this enzyme(s) may be modified by drug administration or by certain disease states such as has been shown in uraemics with 6MP [13].

This paper has characterized CMT activity in a human RBC lysate preparation and has shown a K_m for CP of 0.5 mM and a V_{max} of 0.391 pmoles SMC (mg protein)$^{-1}$ min^{-1}. Comparison with another structurally unrelated sulfhydryl drug, 6MP, suggests that thiol methyltransferase probably shows broad substrate selectivity since both the K_m and V_{max} for 6MP and CP were not greatly different. The lack of substrate specificity of thiol methyltransferases has also been suggested by previous studies in rat liver [14], and mouse and rat kidney [15]. However, it is of interest that endogenous sulfhydryl compounds such as cysteine, glutathione, and even dithioerythritol, are not substrates for this enzyme [14].

The addition of dithioerythritol made little difference to the activity of the enzyme; however, it was routinely included in all incubations since a previous study suggested it may decrease variability between enzyme preparations by restoring some lost activity [4].

Previous studies on human RBC lysates using 6MP as substrate have described a pH optimum for the enzyme at 7.5 [4], the pH used in these experiments and the lack of an effect by both calcium and magnesium ions [5]. Other ions that may potentially regulate the activity of this enzyme have not been studied.

The distribution of CMT in a limited number of rat tissues was surprisingly wide. Brain tissue was not examined here since CP does not cross the 'blood-brain' barrier [16]. Other tissues, such as skin, which may play a role in determining the toxicity of CP were not examined here. The liver contained the highest activity of all the other tissues examined. The order of activity was liver > heart > spleen > lung > kidney ≫ RBC. This order of activity was quite different when 2-thiouracil or 6MP was used as the substrate in rat or mouse soluble fractions

Table 1. Tissue distribution of captopril thiol methyltransferase (CMT) activity*

Tissue	CMT activity [pmoles *S*-methylcaptopril (mg protein)$^{-1}$ min^{-1}]
Human RBC lysates	0.36 ± 0.10
Rat RBC lysates	0.06 ± 0.02
Rat liver	26 ± 11
Rat heart	15 ± 8
Rat spleen	15 ± 9
Rat lung	6.3 ± 2.7
Rat kidney	3.9 ± 1.5

* Mean of four or five experiments on separate preparations for rat tissues and six for human RBC lysates. Errors are expressed as ± S.E.

† 10,000-g_{max}, 10-min supernatant fractions.

[15]. In this study the order of activity was kidney ≫ liver > spleen with little or not activity in lung and heart. Further differences in the tissue distribution of thiol methyltransferase activity was shown by another study in rat homogenates using 2-thioacetanilide as substrate in which the order of activity was liver > lung > kidney > spleen > heart > RBC [17]. Although tests of statistical significance were not conducted by these latter two studies it would appear that these large differences are difficult to be explained simply by the purity of the enzyme preparation. A likely explanation is heterogeneity of thiol methyltransferase enzymes. It is quite possible that two or even a number of these isoenzymes occur with different substrate specificities and variable tissue or subcellular distributions. Evidence for the heterogeneity of thiol methyltransferase enzymes has already been suggested by a membrane-bound form of this enzyme (13,000-g_{max}, 10-min pellet) which catalyses the methylation of 2-mercaptoethanol but not 6MP [5], whereas a 7000-g_{max}, 10-min supernatant fraction catalyses the *S*-methylation of 6MP [4].

These two enzyme preparations probably correspond to a mitochondrial and a microsomal-bound enzyme respectively as shown by the subcellular distribution reported here and is consistent with Bremer and Greenberg's [6] membrane-bound activity that catalyses the methylation of xenobiotics and studies by Weisiger and Jakoby [14].

In contrast, Remy [15] has demonstrated a soluble enzyme located in the 100,000-g_{max} supernatant fraction of both rat and mouse kidney and liver homogenates that catalysed methylation of 6-thiopurine and 2-thiouracil. Further studies are clearly needed on a range of different substrates to determine the possible heterogeneity of thiol methyltransferase.

In addition this paper has shown evidence for regulation of CMT activity by substrate and by the end-product SMC, but not by an oxidation product of CP (disulfide dimer). This is the first evidence to suggest that the activity of this enzyme(s) may be modified by specific compounds. The possibility of endogenous regulatory compounds modifying the activity of CMT cannot be excluded, particularly in cases of uraemia where endogenous toxins are known to accumulate [13].

The significance of CMT activity was assessed by measuring the tissue levels of SMC in rat liver following a 10 mg/kg dose (gavage). At 30 min a SMC level of 1.05 μg/g wet weight was obtained (Drummer *et al.*, unpublished observations). Further studies are in progress to determine whether CMT activity is changed during chronic dosing of captopril in both man and rats and whether variations in CMT activity result in significant variations in the degree of *S*-methylation of CP *in vivo*. This study is particularly relevant to the study of human metabolism of CP since it utilizes a sensitive assay procedure for the measurement of CMT activity in an easily obtained human tissue such as blood. This should make it possible to determine whether the quite large individual variations in the activity of thiol methyltransferase enzymes in RBC lysates [4] might play an important role in causing variations in the effectiveness and toxicity of CP administration.

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REFERENCES

1. R. C. Heel, R. N. Brogden, T. M. Speight and G. S. Avery, *Drugs* **20**, 409 (1980).
2. O. H. Drummer, B. Jarrott and W. J. Louis, *Clin. exp. Pharmac. Physiol.* Suppl. 7, 81 (1982).
3. T. Ikeda, T. Komai, K. Kawai and H. Shindo, *Chem. pharm. Bull. Tokyo* **29**, 1416 (1981).
4. R. M. Weinshilboum, F. A. Raymond and P. A. Pazmino, *Clinica chim. Acta* **85**, 323 (1978).
5. R. M. Weinshilboum, S. Sladek and S. Klumpp, *Clinica chim. Acta* **97**, 59 (1979).
6. J. Bremer and D. M. Greenberg, *Biochim. biophys. Acta* **46**, 217 (1961).
7. R. T. Borchardt and C. F. Cheng, *Biochim. biophys. Acta* **522**, 340 (1978).
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
9. P. J. Munson and D. Rodbard, *Analyt. Biochem.* **107**, 220 (1980).
10. O. H. Drummer and B. Jarrott, *J. Chromat.* (submitted for publication).
11. C. De Duve, in *Enzyme Cytology* (Ed. D. B. Roodyn), p. 1. Academic Press, London (1967).
12. J. Axelrod and C. K. Cohn, *J. Pharmac. exp. Ther.* **176**, 650 (1971).
13. P. A. Pazmino, S. L. Sladek and R. M. Weinshilboum, *Clin. Pharmac. Ther.* **28**, 356 (1980).
14. R. A. Weisiger and W. B. Jakoby, *Archs Biochem. Biophys.* **196**, 631 (1979).
15. C. N. Remy, *J. biol. Chem.* **238**, 1978 (1963).
16. J. S. Hutchinson, R. Hooper, B. Jarrott, F. A. O. Mendelsohn and W. J. Louis, in *Proceedings of the 4th International Symposium on Rats with Spontaneous Hypertension and Related Studies, September* 1981.
17. R. A. Weisiger, L. M. Pinkus and W. B. Jakoby, *Biochem. Pharmac.* **29**, 2885 (1980).