PHENYLALANINE 4-MONOOXYGENASE AND THE *S*-OXIDATION OF *S*-CARBOXYMETHYL-L-CYSTEINE IN HepG2 CELLS

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SUMMARY

The role of phenylalanine 4-monooxygenase (PAH) in the *S*-oxidation of *S*-carboxymethyl-L-cysteine (SCMC) in the rat has now been well established in rat cytosolic fractions *in vitro*. However, the role of PAH in the *S*-oxidation of SCMC in human cytosolic fractions or hepatocytes has yet to be investigated. The aim of this investigation was to analyse the kinetic parameters of PAH oxidation of both L-phenylalanine (Phe) and SCMC in the human HepG2 cell line in order to investigate the use of these cells as a model for the cellular regulation of SCMC *S*-oxidation. The experimentally determined K_m and V_{max} were 7.14 ± 0.32 mM and 0.85 ± 0.32 nmole Tyr formed min^{-1}.mg $protein^{-1}$ using Phe as substrate. For SCMC the values were 25.24 ± 5.91 mM and 0.79 ± 0.09 nmole SCMC (*R/S*) *S*-oxides formed min^{-1}.mg $protein^{-1}$. The experimentally determined K_m and

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V_{max} for the cofactor BH_4 were 6.81 ± 0.21 μM and 0.41 ± 0.004 nmole Tyr formed min^{-1}.mg $protein^{-1}$ for Phe and 7.24 + 0.19 μM and 0.42 ± 0.002 nmole SCMC (*R/S*) *S*-oxides formed min^{-1}.mg $protein^{-1}$ for SCMC. The use of various PAH inhibitors confirmed that HepG2 cells contained PAH and that the enzyme was capable of converting SCMC to its (*R*) and (*S*) *S*-oxide metabolites in an *in vitro* PAH assay. Thus HepG2 cells have become a useful additional tool for the investigation of the cellular regulation of PAH in the *S*-oxidation of SCMC.

KEY WORDS

S-carboxymethyl-L-cysteine, *S*-oxidation, phenylalanine 4-monooxygenase, substrate specificity, enzyme kinetics, HepG2 cells

INTRODUCTION

S-Carboxymethyl-L-cysteine (SCMC) is a muco-regulatory drug used in the treatment of chronic obstructive pulmonary disease (COPD) and otitis media with effusions (OME) to alter mucus viscosity in the lung or inner ear /1,2/. The pharmacodynamic action of SCMC is at present unknown. However, this may include reactive oxygen intermediate scavenging /3/, alteration of mucus composition /4,5/, inhibition of neutrophil activation /6/ and inhibition of the attachment of bacteria to human pharyngeal epithelial cells /7,8/. The metabolism of SCMC in man is also poorly understood. The biotransformation pathways in man have been reported to include deamination of SCMC to thiodiglycolic acid /9/, deamination and *S*-oxidation to thiodiglycolic acid *S*-oxide /10/ and C-S bond lysis of SCMC to produce the *S*-(carboxymethylthio)-L-cysteine disulphide /11/. The original observations that the major metabolites of SCMC in man were in fact *S*-oxides of SCMC and *S*-methyl-L-cysteine (SMC) /12/ have since been proved to be correct /13,14/. The enzyme involved in this biotransformation is under both genetic and hormonal control in man /13,14/ but its identity has still to be elucidated.

It was originally postulated that CYP2D6 was involved /15/ but this was later disproved /16/. Cysteine dioxygenase was also hypothesised to be involved in the *S*-oxidation of SCMC /17/ but this

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was also found not to be the case /18/. To date, the only detailed investigation into the substrate and cofactor/inhibitor specificity requirements of "SCMC *S*-oxygenase" were reported by Khan *et al.* in the rat /18/ and Waring *et al.* in a number of mammalian species /19/. The results from these investigations found that "SCMC *S*-oxygenase" was located in the cytosolic fraction of the liver, required Fe^{2+} for catalytic activity, was activated by sulphydryl modifying reagents, but was inactivated by hydrogen peroxide. Reports in the literature have stated that purified rat phenylalanine 4-monooxygenase (PAH) activated by a number of post-translation mechanisms can carry out the *S*-oxidation of L-methionine (Met) and *S*-methyl-L-cysteine (SMC) /20/. Subsequent investigations with activated rat cytosolic fractions found that PAH could also *S*-oxidise Met, SMC and SCMC /21,22/. Thus the identity of the enzyme involved in the *S*-oxidation of the muco-regulatory drugs SCMC and SMC appears to be resolved.

In the present study we investigated the possibility that PAH is the enzyme responsible for the *S*-oxidation of SCMC in man by using the human hepatoma cell line HepG2 as a possible model for human hepatocytes.

MATERIALS AND METHODS

Materials

Bovine liver catalase, SCMC, L-cysteic acid, deferoxamine, 2,2′-dipyridyl, disodium hydrogen phosphate, lysophospatidylcholine, 1,4-dithiotreitol (DTT), *N*-ethylmaleimide (NEM), glacial acetic acid, hydrogen peroxide (30% w/v), 2-mercaptoethanol (2ME), *o*-phthaldehyde (OPA), sodium acetate, sodium borate, sodium dihydrogen phosphate, sodium hydrogen carbonate and trichloroacetic acid (TCA), were purchased from Sigma-Aldrich Chemical Company, Poole, Dorset, UK. High-pressure liquid chromatography grade methanol was from Rathburn Chemical Company, Walkerburn, Scotland. SCMC (*R*)- and (*S*)-*S*-oxides were synthesised by hydrogen peroxide oxidation of SCMC and isolated by fractional re-crystallization /23/. All other chemicals were readily available in pure form within the laboratory.

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Cell line

HepG2 cells were obtained from the European Collection of Cell Cultures (ECACC). The HepG2 cells were grown under the recommended ECACC optimal growth conditions as follows. HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine plus 10% (v/v) foetal calf serum. In this investigation the cells were incubated in streptomycin- and penicillin-free conditions and the HepG2 cells were maintained as a multilayer in 15 ml of culture medium in 75 cm^2 plastic tissue culture flasks. HepG2 cells grow as clumps and rarely as a single monolayer. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2:95% O_2. Cultured cells were routinely passaged every 7 days at a seeding density of 1 x 10^4 to 5 x 10^4 cells/cm^2 with media changes every 48-72 h. Cells at 70-80% confluence were used in all experiments. The cell passage number was 120-128. Whole cell homogenates were prepared as described previously /24/.

Protein determination

The cytosolic fraction was assayed for protein content by the method of Bradford /25/.

PAH activity using L-phenylalanine (Phe) as substrate

The activated PAH activity in the HepG2 cell cytosolic fraction was investigated using a modification of the method of Kaufman /26/. The assay was performed by the sequential addition of 100 µl of 100 mM Phe, 100 µl of 500 mM potassium phosphate buffer at pH 6.8, 100 µl of catalase (78,000 units/ml), 100 µl of HepG2 cell cytosol (25.0 µg protein/ml), 100 µl of 60 mM DTT solution and 200 µl of 500 µM BH_4 in 5 mM HCl. All concentrations reported here are initial concentrations. The mixture was made up to 1.0 ml with de-ionised water and incubated at 37°C for 30 min. The reaction was terminated by the addition of 2 ml of 12% (w/v) TCA solution. The precipitated protein was removed by centrifugation at 1,800 *g* for 10 min. The clear supernatant (containing the reaction product L-tyrosine [Tyr]) was removed and analysed by the method of Kaufman /26/. The

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blanks contained the same components as the test sample but used heat-inactivated HepG2 cell cytosol instead.

PAH activity using SCMC as substrate

This assay was identical to that which used Phe as substrate with the following exception: 100 µl of 500 mM SCMC replaced 100 mM Phe. The blanks contained the same components as the test sample but used heat-inactivated HepG2 cell cytosol instead. The assay was terminated by the addition of 100 µl of 20% (w/v) TCA solution. The internal standard (2 µl of 10 mM cysteic acid solution) was added. The sample was centrifuged, derivatized and analysed by hplc with fluorescence detection.

Determination of SCMC (R/S) *S*-oxides by hplc

The separation and quantification of SCMC (*R*) and (*S*) S-oxides by pre-column derivatization with OPA/2ME was carried out as described previously /27/.

Solid-phase extraction

Chromabond® SA (100 mg bed weight) solid phase extraction columns (SPE) were conditioned with 5 ml methanol then 5 ml methanol/0.1 M HCl (1:1, v/v), and finally 5 ml of 0.1 M HCl using the Visiprep™ solid phase extraction apparatus (Supelco). 1 ml of supernatant was applied to the Chromabond® SA SPE columns. The SPE columns were then eluted with 1 ml of H_2O. The eluant was then neutralised with 1 M NaOH.

Pre-column derivatization

OPA/2ME derivatizing reagent was prepared as follows: 22.0 ml of 100 mM sodium borate buffer (pH 9.5) was added to 18.0 ml of *o*-phthaldehyde/2-mercaptoethanol solution (144 mg of OPA in 18.0 ml of methanol containing 90 µl of 2ME). This is prepared daily and protected from light in a brown glass reagent bottle. 90 µl of the derivatization mixture was then added to 20 µl of standard/sample by the TSP AS3500 pre-column derivatizing autosampler. Both preparation and sample syringe were washed out before each injection with

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water:methanol mixture (50:50 v/v). This procedure took a total of 2 min.

High pressure liquid chromatography apparatus

The system consisted of a Waters 600E multi-solvent delivery pump connected to a TSP AS3500 pre-column derivatizing auto-sampler. The fluorescence detector was a LaChrome (Merck-Hitachi) L-7480 model with the PMT set to low and data capture was by the "Prime high-pressure liquid chromatography data capture software" (Hplc Technology Ltd., UK).

Analytical column

5 µl of the standard/sample-derivatization solution was injected onto a Hypersil Amino Acid 5µ C_{18} (30 x 2.1 mm i.d.) analytical column protected by a Hypersil Amino Acid guard column. SCMC, L-cysteic acid (IS) and SCMC *R* and *S S*-oxides were separated by a gradient elution. Solvent A was 90% v/v (50 mM sodium acetate, pH 5.5) and methanol (10% v/v) and solvent B was 100% (v/v) methanol. The analytical column was eluted isocratically with 100% A (0-5 min) at a flow rate of 0.2 $ml.min^{-1}$ followed by a linear gradient to 5% A:95% B (5.0-6.5 min) at a flow rate of 0.5 $ml.min^{-1}$. This was held isocratically at 5% A:95% B (6.5-11.5 min) at a flow rate of 0.5 $ml.min^{-1}$ before a second linear gradient to 100% A was initiated (14.0-16.0 min) at a flow rate of 0.2$ml.min^{-1}$. The column temperature was 25°C and the excitation and emission wavelengths were 344 and 443 nm.

Data analysis

Enzyme kinetic data were analysed by the Leonora enzyme kinetic programme version 1.0 /21/ using the Michaelis-Menten equation, $V = V.[S]/K_m + [S]$, where V = velocity, S = substrate, K_m = Michaelis-Menten constant, and the Michaelis-Menten with substrate inhibition equation, $V = V.[S]/(K_s + [S](1 + [S]/K_{si}))$, where V = velocity, S = substrate, K_s = Michaelis-Menten constant for substrate when substrate inhibition was present and K_{si} = inhibitor constant for the substrate.

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RESULTS

A chromatogram of the separation of SCMC, SCMC (*R*) and (*S*) *S*-oxides and L-cysteic acid (IS) can be seen in Figure 1. Baseline separation was achieved for SCMC, L-cysteic acid and SCMC (*R*) and (*S*) *S*-oxides. The effects of both time and cytosolic protein concentration on PAH activity with respect to the production of Tyr (from Phe) were investigated. Both variables were found to have a linear relationship against product formation for time (5-60 min) (Fig. 2a) and protein concentration (5-30 μg/ml) (Fig. 2b).

The effects of varying Phe concentration at a fixed cofactor (BH_4) concentration (100.0 μM) can be seen in Figure 3a. The V against S plot shows that the data followed the Michaelis-Menten equation with substrate inhibition (solid line) compared to the Michaelis-Menten equation (dashed line). The Leonora calculated K_m and V_{max} values for the data in Figure 3a were 7.14 ± 0.32 mM and 0.85 ± 0.32 nmole Tyr formed min^{-1}.mg $protein^{-1}$ for Phe (Table 1). The effects of varying the cofactor (BH_4) concentration at a fixed substrate concentration (10.0 mM) can be seen in Figure 3b. The V against S plot clearly shows that the data followed the Michaelis-Menten equation with substrate inhibition (solid line) compared to the Michaelis-Menten equation (dashed line). The Leonora calculated K_m and V_{max} values for the data in Figure 3b were 6.81 ± 0.21 μM and 0.41 ± 0.004 nmole Tyr formed min^{-1}.mg $protein^{-1}$ for Phe (Table 2).

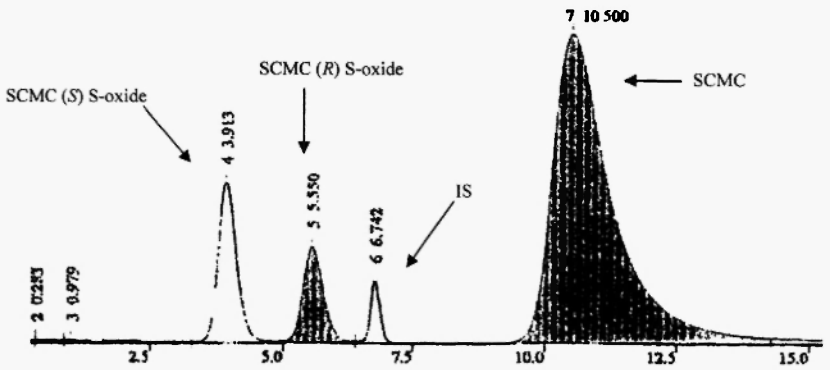

Fig. 1: Chromatogram of the separation of 5 μM *S*-carboxymethyl-L-cysteine (SCMC), SCMC (*R*) *S*-oxide, SCMC (*S*) *S*-oxide and L-cysteic acid (IS).

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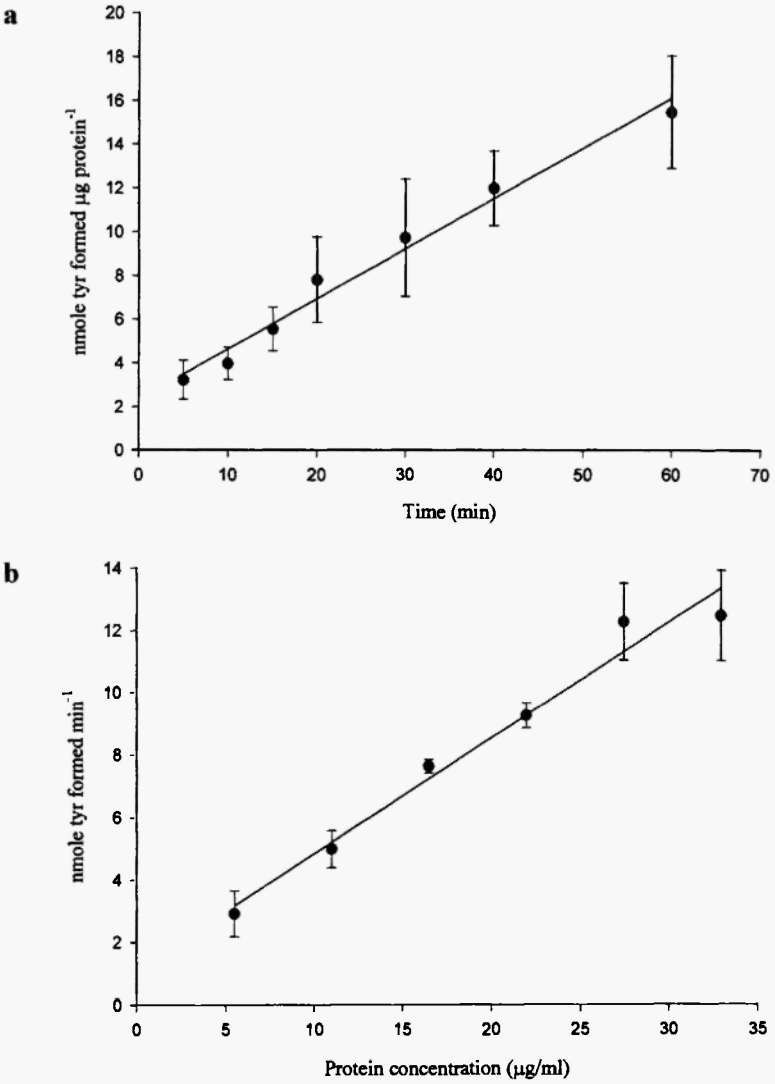

Fig. 2: **a.** Linearity of tyrosine (tyr) production versus time for "activated" HepG2 cell cytosolic fraction phenylalanine 4-monooxygenase (PAH) activity with BH_4 concentration set at 100.0 µM, L-phenylalanine (Phe) at 10 mM and protein concentration at 25.0 µg/ml. Each value is the mean ± SD of 6 experiments. **b.** Linearity of tyrosine (tyr) production versus protein concentration for "activated" HepG2 cell cytosolic fraction phenylalanine 4-monooxygenase (PAH) activity with BH_4 concentration was set at 100.0 µM, Phe at 10 mM and time at 30 min. Each value is the mean ± SD of 6 experiments.

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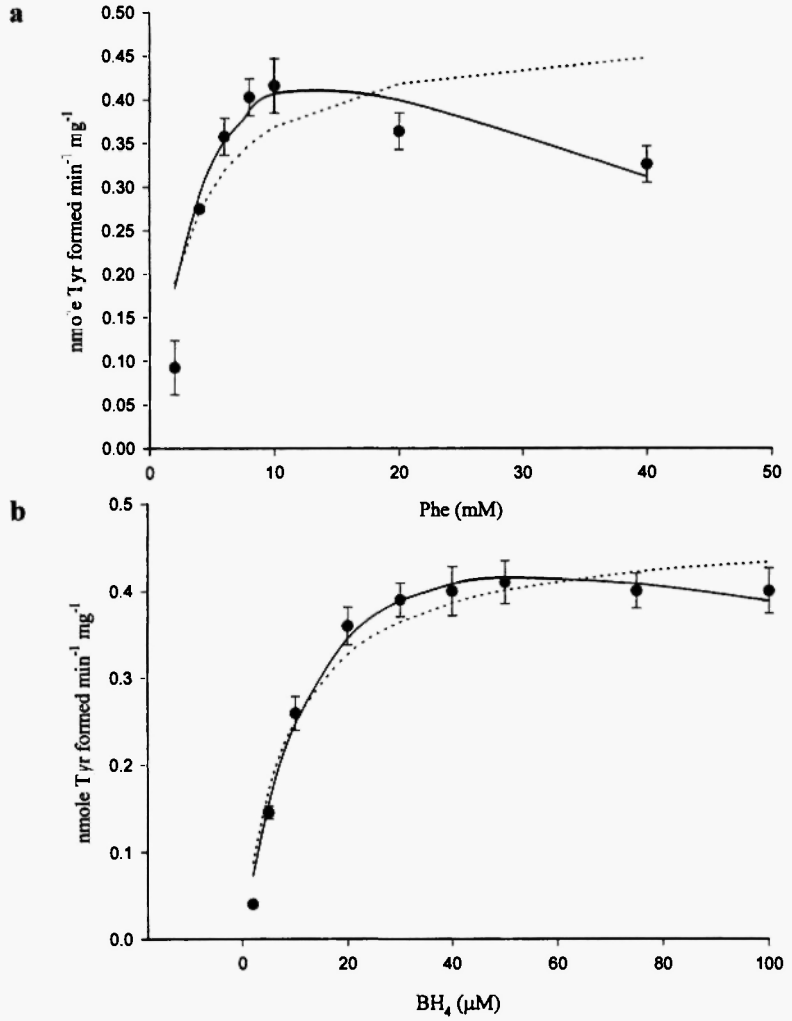

Fig. 3: a. The V against S plots of varying Phe substrate concentrations with BH_4 (cofactor) concentration set at 100.0 μM for "activated" HepG2 cell cytosolic fraction phenylalanine 4-monooxygenase (PAH) activity. The solid line is the Michaelis-Menten with substrate inhibition equation fit to the experimental data. The dashed line is the Michaelis-Menten equation fit to the experimental data. Each value is the mean ± SD of 6 experiments. b. The V against S plots of varying BH_4 (cofactor) concentrations with Phe substrate concentration set at 10.0 mM for "activated" HepG2 cell cytosolic fraction phenylalanine 4-monooxygenase (PAH) activity. The solid line is the Michaelis-Menten with substrate inhibition equation fit to the experimental data. The dashed line is the Michaelis-Menten equation fit to the experimental data. Each value is the mean ± SD of 6 experiments.

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TABLE 1

K_m, V_{max} and K_i data for activated phenylalanine 4-monooxygenase (PAH) assays using L-phenylalanine (Phe) and *S*-carboxymethyl-L-cysteine (SCMC) as substrates or inhibitors

Substrate	**n**	**K_m** (mM)	**V_{max}** (nmoles product formed min^{-1}.mg cytosolic $protein^{-1}$)	**K_i** (mM)
Phe	6	7.14 ± 0.32	0.85 ± 0.32	6.58 + 0.50*
SCMC	6	25.24 ± 5.91	0.79 ± 0.09	22.60 ± 6.51$^+$

Leonora 1.0 calculated K_m, V_{max} and K_i values for "activated" HepG2 cell cytosolic fraction PAH activity using varying Phe and SCMC substrate concentrations with BH_4 (cofactor) concentration set at 100.0 μM. Each value is the mean ± SD of 6 experiments.

n = number of experiments. Each experiment was determined in duplicate with associated blanks.

PAH was activated by pre-incubation with 1.0 mM lysophosphatidylcholine for 3.0 min at 37°C.

* Using Phe as an inhibitor of SCMC *S*-oxidation.

$^+$ Using SCMC as an inhibitor of Phe *C*-oxidation.

TABLE 2

K_m and V_{max} data for the cofactor BH_4 in activated phenylalanine 4-monooxygenase (PAH) assays using phenylalanine 4-monooxygenase (PAH) and *S*-carboxymethyl-L-cysteine (SCMC) as substrates

Substrate	**n**	**K_m** (μM)	**V_{max}** (nmoles product formed min^{-1}.mg cytosolic $protein^{-1}$)
Phe	6	6.81 ± 0.21	0.41 ± 0.004
SCMC	6	7.24 ± 0.19	0.42 ± 0.002

Leonora 1.0 calculated K_m and V_{max} values for "activated" HepG2 cell cytosolic fraction PAH activity using 10.0 mM Phe and 50.0 mM SCMC substrate concentrations with varying BH_4 (cofactor) concentration. Each value is the mean ± SD of 6 experiments.

n = number of experiments. Each experiment was determined in duplicate with associated blanks.

PAH was activated by pre-incubation with 1.0 mM lysophosphatidylcholine for 3.0 min at 37°C.

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The results of the investigations into the effects of both time and cytosolic protein concentration on PAH activity with respect to the production of SCMC (*R/S*) *S*-oxides (from SCMC) were investigated. Both variables were found to have a linear relationship against product formation for time (5-60 min) (Fig. 4a) and protein concentration (5-30 μg/ml) (Fig. 4b).

The effects of varying SCMC concentration at a fixed cofactor (BH_4) concentration (100.0 μM) can be seen in Figure 5a. The V against S plot shows that the data for SCMC followed the Michaelis-Menten equation with substrate inhibition (solid line) compared with the Michaelis-Menten equation (dashed line). The Leonora calculated K_m and V_{max} values for the data in Figure 5a were 25.24 ± 5.91 mM and 0.79 ± 0.09 nmole SCMC (*R/S*) *S*-oxides formed min^{-1}.mg $protein^{-1}$ (Table 1). The effects of varying the cofactor (BH_4) concentration at a fixed substrate concentration (50.0 mM) can be seen in Figure 5b. The V against S plot shows that the data can be modelled by both the Michaelis-Menten equation with substrate inhibition (solid line) and the Michaelis-Menten equation (dashed line). The Leonora calculated K_m and V_{max} values for the data in Figure 5b using the Michaelis-Menten equation were 7.24 ± 0.19 μM and 0.42 ± 0.002 nmole SCMC (*R/S*) *S*-oxides formed min^{-1}.mg $protein^{-1}$ for SCMC (Table 2).

The Leonora calculated K_i constants for SCMC and Phe can be seen in Table 1. The K_i for SCMC acting as an inhibitor of PAH activity when Phe was used as the substrate was 22.60 ± 6.51 mM. The Leonora predictive mode of inhibition for SCMC was competitive inhibition. The K_i for Phe acting as an inhibitor of PAH activity when SCMC was used as the substrate was 6.58 ± 0.50 mM. The Leonora predictive mode of inhibition for Phe was competitive inhibition (Table 1). The effects of various known inhibitors of PAH activity were investigated using Phe and SCMC as substrates (Table 3). The thioether substrates Met, SCMC and SMC at a concentration of 5.0 mM were all found to inhibit Phe *C*-oxidation. Met was the more potent of the thioether inhibitors used causing 39% inhibition. SCMC resulted in 28.5% inhibition and SMC 11% inhibition. When SCMC was used as the substrate, 5.0 mM Met resulted in 18.8% inhibition of SCMC *S*-oxidation, 5.0 mM SMC caused 7.9% inhibition but Phe (5.0 mM) resulted in >99% inhibition of SCMC *S*-oxidation. The large

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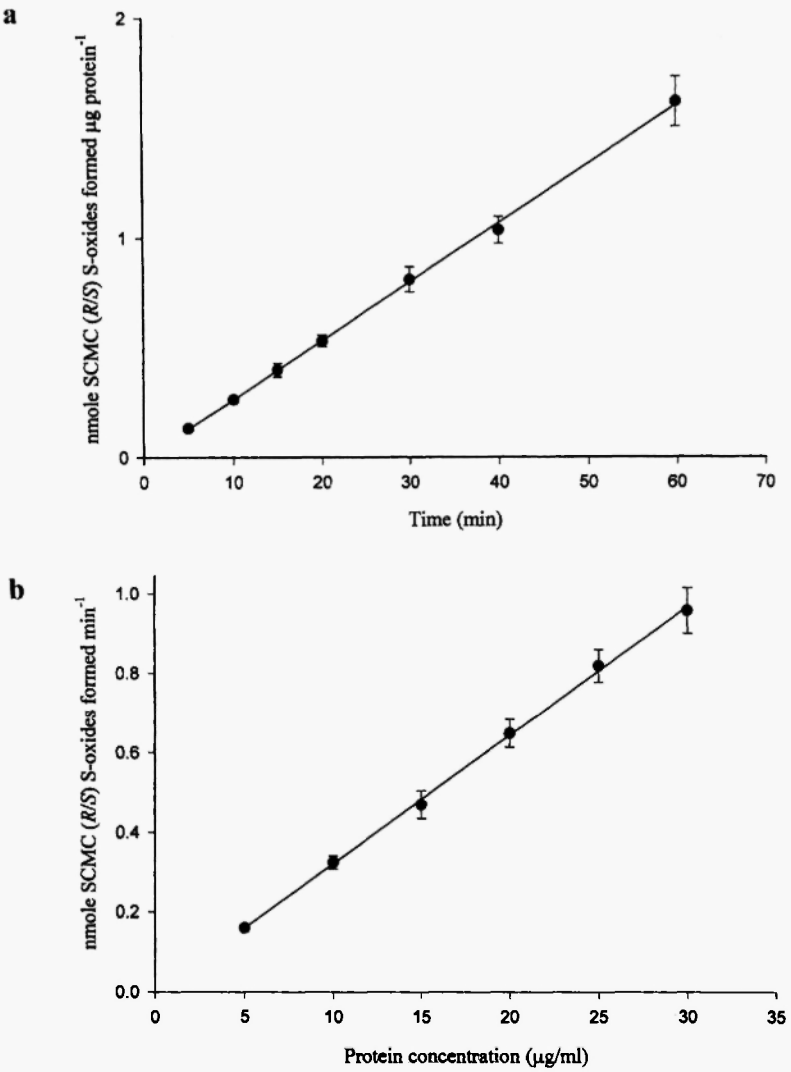

Fig. 4: **a.** Linearity of *S*-carboxymethyl-L-cysteine (SCMC) (*R/S*) *S*-oxides production versus time for "activated" HepG2 cell cytosolic fraction phenylalanine 4-monooxygenase (PAH) activity with BH_4 concentration set at 100.0 µM, SCMC at 50 mM and protein concentration at 25.0 µg/ml. Each value is the mean ± SD of 6 experiments. **b.** Linearity of *S*-carboxymethyl-L-cysteine (SCMC) (*R/S*) *S*-oxides production versus protein concentration for "activated" HepG2 cell cytosolic fraction phenylalanine 4-monooxygenase (PAH) activity with BH_4 concentration set at 100.0 µM, SCMC at 50 mM and time at 30 min. Each value is the mean ± SD of 6 experiments.

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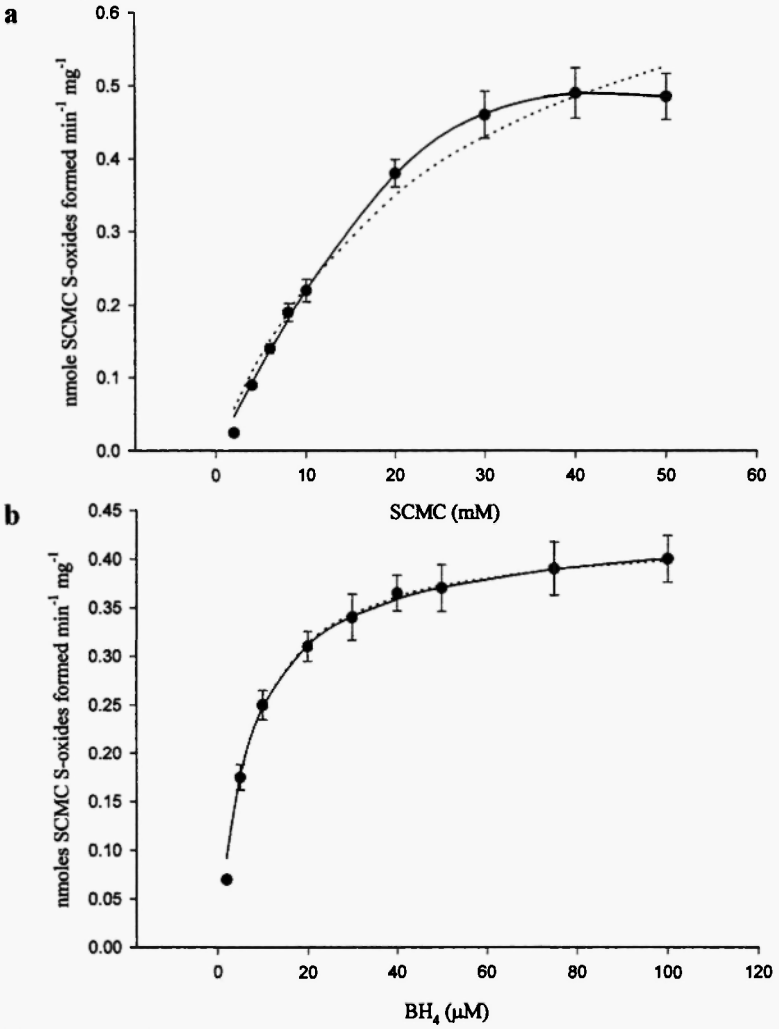

Fig. 5: a. The V against S plots of varying *S*-carboxymethyl-L-cysteine (SCMC) substrate concentrations with BH_4 (cofactor) concentration set at 100.0 μM for "activated" HepG2 cell cytosolic fraction phenylalanine 4-monooxygenase (PAH) activity. The solid line is the Michaelis-Menten with substrate inhibition equation fit to the experimental data. The dashed line is the Michaelis-Menten equation fit to the experimental data. Each value is the mean ± SD of 6 experiments. **b.** The V against S plots of varying BH_4 (cofactor) concentrations with *S*-carboxymethyl-L-cysteine (SCMC) substrate concentration set at 50.0 mM for "activated" HepG2 cell cytosolic fraction phenylalanine 4-monooxygenase (PAH) activity. The solid line is the Michaelis-Menten with substrate inhibition equation fit to the experimental data. The dashed line is the Michaelis-Menten equation fit to the experimental data. Each value is the mean ± SD of 6 experiments.

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TABLE 3

Effects of various inhibitors on activated phenylalanine 4-monooxygenase (PAH) activity using L-phenylalanine (Phe) and *S*-carboxymethyl-L-cysteine (SCMC) as substrates

Compound (concentration)	**n**	**% of control activity**	
		Phe as substrate	**SCMC as substrate**
Met (5.0 mM)	6	61.0 + 5.0	81.2 ± 3.5
SMC (5.0 mM)	6	89.0 ± 6.0	92.1 ± 5.6
SCMC (5.0 mM)	6	71.5 ± 4.8	ND
Phe (5.0 mM)	6	ND	0.2 ± 0.3
MCA (200 μg/ml)	6	<0.1	<0.1
Deferoxamine (1.0 mM)	6	1.8 ± 0.5	0.8 ± 0.7
2,2′-Dipyridyl (1.0 mM)	6	<0.1	<0.1

The effects of various inhibitors on "activated" HepG2 cell cytosolic fraction PAH activity using 10.0 mM Phe and 50.0 mM SCMC substrate concentrations and 50.0 μM BH_4 (cofactor) concentration. Each value is the mean ± SD of 6 experiments.
Met = L-methionine; SMC = *S*-methyl-L-cysteine; MCA = aromatic amino acid hydroxylase monoclonal antibody.
n = number of experiments. Each experiment was determined in duplicate with associated blanks.
PAH was activated by pre-incubation with 1.0 mM lysophosphatidylcholine for 3.0 min at 37°C.

aromatic amino acid hydroxylase monoclonal antibody (200 μg/ml) resulted in >99% inhibition of both Phe and SCMC metabolism (Table 3). The two iron chelators, deferoxamine (1.0 mM, Fe^{3+} chelator) and 2,2′-dipydridyl (1.0 mM, Fe^{2+} chelator) caused 98.2% and >99% inhibition of Phe *C*-oxidation, while the same compounds resulted in >99% inhibition of SCMC *S*-oxidation (Table 3).

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DISCUSSION

The study of the enzymology of SCMC *S*-oxidation has received little experimental investigation over the last 18 years. However, the role of *S*-oxidation polymorphism as a disease aetiology factor /28/ has resulted in a number of renewed investigations into the enzyme(s) involved in this biotransformation reaction. Recent investigations in post-translational activated and non-activated Wistar rat cytosolic fractions have implicated PAH as the enzyme responsible for the *S*-oxidation of SCMC /21,22,29/. Although the use of subcellular fractions *in vitro* provides investigators with the ability to conduct reaction phenotyping investigations (correlation studies, inhibitor studies, substrate specificity studies), the lack of the "whole cell" as an integrated model of cellular function has its limitations (lack of regulation at the gene level). Thus the use of primary cells or immortalised cell lines in culture provides investigators with this integrated cellular response. The presence of PAH activity in the HepG2 cell line /24,30,31/ together with the report that HepG2 cells do not contain cysteine dioxygenase activity /32/ means that this cell line has the potential to be used as a model for *in vivo* hepatic *S*-oxidation of SCMC in man.

HepG2 cytosolic fractions were used in this initial investigation to compare the *in vitro* kinetic parameters of PAH activity with those previously reported in the rat /21,22,29/. The production of both Tyr and SCMC (*R/S*) *S*-oxides was linear versus time and HepG2 cell cytosolic protein concentration. The K_m for Phe was higher than in a previous report /30/, but the V_{max} value cannot be compared since no previous literature report could be found. However, the K_m for Phe in the HepG2 cell cytosolic fractions was 27.46- and 17.85-fold higher than in the activated and non-activated rat cytosolic fractions /21,22/. The V_{max} for Phe in the HepG2 cell cytosolic fractions was 22.8% and 9.0% of the values reported for activated and non-activated rat cytosolic fractions /21,22/. This should be compared to the report in the literature that HepG2 cells contain 10-15% of the level of PAH observed in normal human liver /24,33/.

The results from the kinetic investigation of SCMC *S*-oxidation provide a similar story. The K_m for SCMC in the HepG2 cell cytosolic fractions was 1.71- and 5.28-fold higher than in the activated and non-activated rat cytosolic fractions /21,22/. The V_{max} value for SCMC in

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HepG2 cell cytosolic fractions was 1.6% and 51.9% of the values reported in the activated and non-activated rat cytosolic fractions /21,22/. Thus there appear to be quantitative differences in the K_m and V_{max} parameters for both Phe and SCMC between both activated and non-activated rat cytosolic fractions as well as activated HepG2 cell cytosolic fractions *in vitro*.

The requirement for the cofactor BH_4 in the *in vitro* enzyme assays, together with the use of known substrates (Met, SMC, SCMC and Phe) of PAH to inhibit the *C*- and *S*-oxidation of Phe and SCMC also provides strong experimental support for the role that PAH plays in the *S*-oxidation of SCMC in HepG2 cells *in vitro* (Table 3). These observations are also supported by the fact that the large aromatic amino acid hydroxylase monoclonal antibody and the Fe^{2+}/Fe^{3+} chelators completely inhibited PAH *C*- and *S*-oxidation of Phe and SCMC in the enzyme assays. These results indicate that the HepG2 cell line contains PAH that can be activated by post-translational modification *in vitro*. This cell line also has the potential for studying the *S*-oxidation of SCMC in culture utilising activation of the PAH gene at the transcriptional level along with the PAH protein at the post-transcriptional level. However, the use of immortalised lines does not necessarily indicate that these mechanisms are at work *in vivo*. Further studies using primary human hepatocytes will also be required to confirm the observations in HepG2 cells.

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