INHIBITION OF RAT RENAL C-S LYASE: ASSESSMENT USING KIDNEY SLICE METHODOLOGY

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CONTENTS

SUMMARY

Renal C-S lyase enzymes are implicated in the biotransformation of xenobiotics into potentially toxic metabolites by a deviation from the normal pathways of glutathione conjugate processing. C-S lyase enzymes occur in gastro-intestinal bacteria, and in liver as well as in mammalian and avian kidney. The renal enzyme cleaves the carbon-sulphur bond in cysteine conjugates derived from halogenated olefins (e.g. tetrafluoroethene, trichloroethene, and hexachlorobutadiene). Substituted S-nitrophenyl conjugates, which are analogues of a substrate for the hepatic C-S lyase enzyme (S-2,4-dinitrophenyl-L-cysteine), are demonstrated to display significant inhibition of rat renal C-S lyase using kidney slice methodology. They are also shown to disrupt the tubular uptake of organic cations and anions.

I. INTRODUCTION

Renal C-S lyase enzymes are involved in the biotransformation of halogenated olefins into toxic and potentially toxic metabolites by a deviation from the normal pathways of glutathione conjugate processing. C-S lyase enzymes are found in human /1/ and other mammalian tissue /2/ and in avian kidney /3/. The renal enzymes are implicated in certain fatal disease states in cattle /4/ and the discovery of toxic effects following the ingestion of trichloroethene (TCE) extracted soy-bean oil meal indicated that the glutathione detoxification pathway had been diverted to form the toxin. The processing of the TCE-glutathione conjugate to S-(E-1,2-dichloroethenyl)-L-cysteine (1) [formerly S-(E-1,2-dichlorovinyl)-L-cysteine (DCVC)] /5/ was found to follow the predicted fate for such xenobiotics (Figure 1). Thus, DCVC is a pro-toxin, the immediate *in vivo* precursor of the reactive moiety /6/.

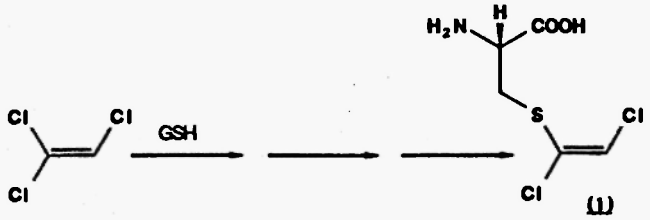


Fig. 1: The metabolism of trichloroethene to the cysteine conjugate S-1,2-dichloroethenyl-L-cysteine.

These observations have been confirmed by closely related studies using hexachloro-1,3-butadiene /7, 8/. This chlorinated olefin is biotransformed to S-1,2,3,4,4-pentachlorobuta-1,3-dienyl-L-cysteine (2) (Figure 2) which may be N-acetylated to afford the corresponding mercapturic acid (3). Rat renal C-S lyase cleaves L-cysteine conjugate substrates producing a thiol which elicits site specific necrosis in the *pars recta* region of the renal proximal tubule /9/; pyruvic acid and ammonia are also produced stoichiometrically with the reactive thiol (Figure 3).

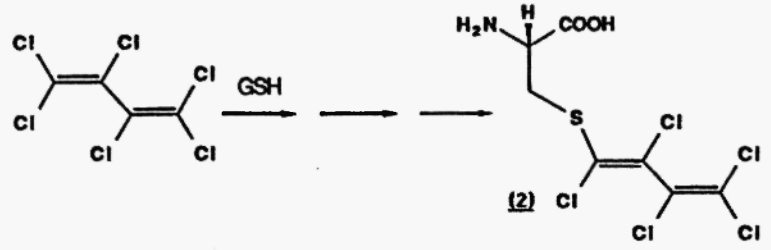


Fig. 2: The metabolism of hexachlorobuta-1,3-diene to the cysteine conjugate S-l,2,3,4,4-pentachlorobuta-1,3-dienyl-L-cysteine.

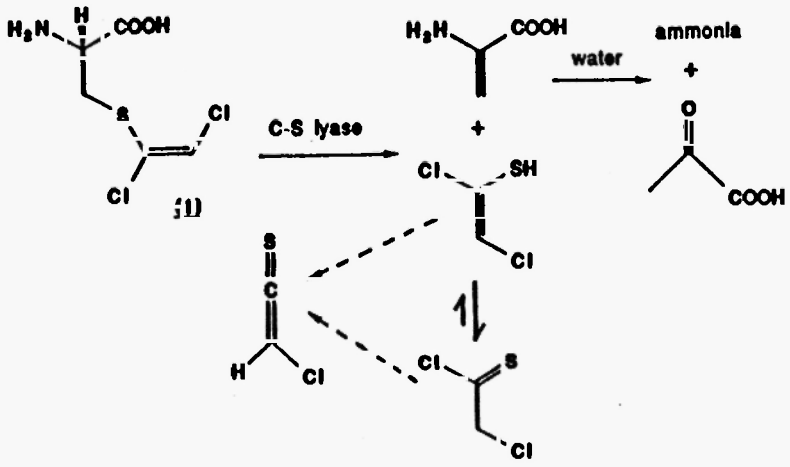
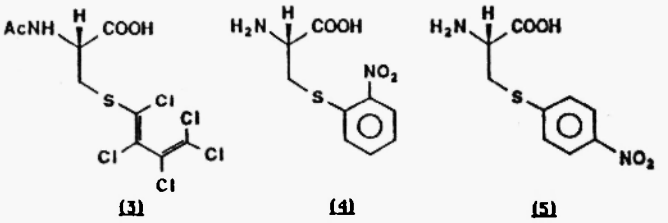


Fig. 3: The C-S lyase enzyme catalysed biotransformation of S-1,2-dichloro-ethenyl-L-cysteine to 2-aminopropenoic acid and 2-chlorothioacetyl chloride.

This thiol may be a precursor for a putative thioketene – possibly the ultimate nephrotoxin. Such a reactive acylating moiety could arise following the elimination of hydrogen chloride from the enethiol or from the thioacetyl chloride after tautomerisation (see Figure 3).

The *in vitro* inhibition data of a series of substituted phenyl-L-cysteine conjugates, with partially purified rat renal C-S lyase, have been previously reported /10/. The inhibition of cytosolic kidney C-S lyase activity by nitrobenzenoid substituted L-cysteine conjugates was determined by monitoring turnover of the established substrate S-1,1,2,2-tetrafluoroethyl-L-cysteine /9/. The concentration of each inhibitor required to elicit a 50% reduction in enzyme activity (IC_{50}) was as follows: 2-nitrophenyl- (4) (1.8 mM), 4-nitrophenyl- (5) (0.5 mM), 2,4-dinitrophenyl- (6) (0.4 mM), and 2-chloro-4-nitrophenyl-L-cysteine (7) (0.6 mM).



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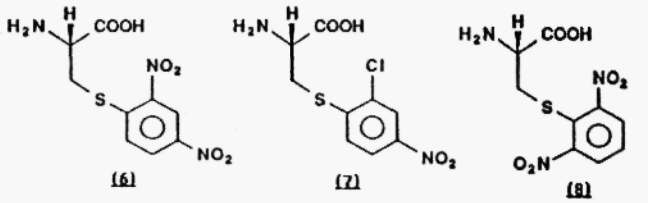

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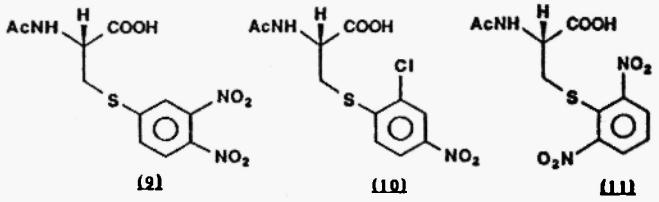

(9) (10) (11)

II. MATERIALS AND METHODS

2.1 Synthesis of Cysteine Conjugates

2.1.1 **S-(2-Nitrophenyl)-L-cysteine (4).** To a solution of L-cysteine (1.03 g, 8.46 mmol) in distilled anhydrous liquid ammonia (20 mL) was added clean sodium metal (17 mmol). After stirring the solution for 40 min, 1-chloro-2-nitrobenzene (1.33 g, 8.42 mmol) was added dropwise during 20 min. The reaction mixture was stirred for an additional 1 hour when the ammonia was removed by evaporation at 25°C. Final traces of ammonia were removed *in vacuo* and water (20 mL) was then added. Extraction of the aqueous solution with diethyl ether (4 x 10 mL) and then adjustment of the pH of the aqueous phase from pH 12 to pH 5 with glacial acetic acid (1 mL) yielded a yellow precipitate. Recrystallisation (EtOH:H_2O, 6:4 v/v; decolourising charcoal, 0.5 g) yielded a yellow amorphous powder (1.02 g, 50%), m.p. 159-160°C, $[\alpha]_D^{24}$ = -118.7° (c = 0.131 in H_2O) λ_{max} (H_2O): 362 (2 868), 272 (3 710), and 240 (13 026) nm, R_F [EtOH:NH_3 (25% w/w in H_2O), 7:3 v/v]: 0.37.

2.1.2 **S-(4-Nitrophenyl)-L-cysteine (5).** Prepared according to the same procedure as for S-(2-nitrophenyl)-L-cysteine except that the following quantities were used: L-cysteine (0.99 g, 8.17 mmol) and 1-chloro-4-nitrobenzene (1.28 g, 8.13 mmol). Recrystallisation (EtOH:H_2O, 8:2 v/v; decolourising charcoal, 0.5 g) yielded a yellow amorphous powder (0.60 g, 30%), m.p. 151-152°C, $[\alpha]_D^{24}$ = -36.9° (c = 0.166 in H_2O), λ_{max} (H_2O): 342 (3 864) nm, R_F [EtOH:NH_3 (25% w/w in H_2O), 7:3 v/v]: 0.37.

2.1.3 **S-(2,6-Dinitrophenyl)-L-cysteine (8).** To anhydrous methanol (150 mL) was added L-cysteine (1.50 g, 12.38 mmol) and then clean sodium metal (25 mmol). 1-Chloro-2,6-dinitrobenzene (2.51 g, 12.4 mmol) was added and the reaction mixture stirred at 25°C for 24 hours. Removal of the methanol *in vacuo* was followed by resuspension of the solid residue in water (50 mL). Extraction of the aqueous suspension with ethyl acetate (4 x 25 mL) and then acidification with glacial acetic acid (2 mL) from pH 9 to pH 5 caused a yellow precipitate to form. The reaction mixture was filtered and the product recrystallised (EtOH:H_2O, 4:1 v/v; decolourising charcoal, 0.5 g) which yielded a yellow amorphous powder (1.8 g, 51%), m.p.

159-160°C (dec.), $[\alpha]_D^{24}$ = -7.4° (c = 0.080 in H_2O), λ_{max} (2M HCl): 319 (1 290) and 230 (10 742) nm.

2.1.4 **N-Acetyl-S-(3,4-dinitrophenyl)-L-cysteine (9).** To a stirred solution of N-acetyl-L-cysteine (1.63 g, 10.0 mmol) in methanol (20 mL), containing 10 M aqueous sodium hydroxide solution (2.0 mL, 20.0 mmol), was added a solution of 1-chloro-3,4-dinitrobenzene (2.43 g 12.0 mmol) in methanol (20 mL). After 15 min, water (300 mL) was added and the solution was extracted with diethyl ether (4x40 mL) and then acidified to pH 1 with concentrated hydrochloric acid (3 mL). The solution was saturated with solid sodium chloride and the mixture then extracted with chloroform (6x100 mL). Evaporation of the pooled, dried, chloroform extracts afforded a solid residue which was recrystallised (EtOH:H_2O, 1:1 v/v) and yielded a light-brown, fluffy, crystalline product (2.46 g, 75%), m.p. 173-174°C, $[\alpha]_D^{24}$ = -9.4° (c = 0.060 in H2O), λ_{max} (MeOH + 1% v/v HOAc): 356 (3 313) and 247 (15 789) nm.

2.1.5 **N-Acetyl-S-(2-chloro-4-nitrophenyl)-L-cysteine (10).** Prepared according to the same procedure as for N-acetyl-S-(3,4-dinitrophenyl)-L-cysteine except that the following quantities were used: N-acetyl-L-cysteine (1.63 g, 10.0 mmol) and 3,4-dichloro-nitrobenzene (2.32 g, 12.0 mmol). Recrystallisation (EtOH:H2O, 1:1 v/v) yielded a yellow, fluffy, crystalline product (2.94 g, 92%), m.p. 187-188°C, $[\alpha]_D^{24}$ = -11.4° (c = 0.100 in H_2O), λ_{max} (MeOH + 1% v/v HOAc): 331 (11 409) and 226 (5 017) nm.

2.1.6 **N-Acetyl-S-(2,6-dinitrophenyl)-L-cysteine (11).** Prepared according to the same procedure as for N-acetyl-S-(3,4-dinitrophenyl)-L-cysteine except that the following quantities were used: N-acetyl-L-cysteine (1.63 g, 10.0 mmol) and 1-chloro-2,6-dinitrobenzene (2.43 g, 12.0 mmol). Recrystallisation (EtOH:H_2O, 1:1 v/v) yielded a yellow crystalline product (2.72 g, 83%), m.p. 177-178°C, $[\alpha]_D^{24}$ = -8.5° (c = 0.120 in H2O), λ_{max} (MeOH + 1% v/v HOAc): 322 (1 160) and 228 (8 908) nm.

2.2 Rat renal cortical slice methodology

The effect of the above substituted phenyl-L-cysteine conjugates on C-S lyase activity was assessed using rat renal cortical slice methodology /11, 12/.

Male, Alderley Park rats (230-260 g) were sacrificed by exposure to an excess of halothane anaesthetic and their kidneys were removed into modified Krebs-Ringer buffer (pH 7.5) maintained at 4°C. The Krebs-Ringer solution was prepared from the following:- sodium chloride (9.00 g, 0.154 mol), potassium chloride (2.65 g, 0.035 mol), magnesium sulphate (19.1% w/v, 2 mL, 77.5 mmol), 0.1 M sodium phosphate buffer (pH 7.4, 100 mL), sodium acetate (0.82 g, 13.2 mmol), and glucose (1.98 g, 11 mmol) in water (to 980 mL). The pH was adjusted to 7.5 (1 M NaOH) before the addition of calcium chloride (6.1% w/v, 2 mL) and then further dilution with water to a final volume of 1 L.

The kidneys were dissected free of their capsule and extraneous fat, cut longitudinally into lateral slices and then stored in modified Krebs-Ringer buffer (pH 7.5, 4°C). Thin renal cortical slices (0.3-0.4 mm, 20-50 mg) were prepared by hand using an 11 cm microtome surgical blade (Gillette valet strip); only slices with two cut surfaces were used in this study. These slices were pre-incubated in the modified buffer (pH 7.5, 2.7 mL) for 15 min at 30°C. All determinations were performed in triplicate.

Kidney slices were incubated for 3 hours at 30°C in an atmosphere of oxygen (100%) with shaking (70 cycles/minute) containing a solution of *P*-amino-[^{3}H]-hippuric acid (Amersham International plc, Amersham, UK; specific activity 374 mCi/mmol), [1-^{14}C]-tetraethylammonium bromide (New England Nuclear Corporation, Boston, USA; specific activity 4.5 mCi/mmol), and N-acetyl-S-pentachlorobuta-1,3-dienyl-L-cysteine (NAcPCBC) (3) (0.5mM) as the substrate (dissolved in dimethyl sulphoxide, 0.06 mL). The solution of PAH and TEA contained Krebs-Ringer buffer (pH 7.5, 8.32 mL), ^{1}H-PAH (21.6 mg/10 mL H_2O, 0.68 mL), ^{3}H-PAH (0.0432 mL), and ^{14}C-TEA (0.0432 mL).

A series of substituted S-phenyl-L-cysteine conjugates was incubated at the following concentrations: 0.0625, 0.125, 0.25, and 0.5mM. Radioactivity in the slice (S) or medium (M) was determined after complete dissolution of the kidney slices (with gentle warming) in Soluene (1 mL) and using Dimulume-30 (10 mL) as the scintillant.

To aliquots of the medium (2x0.1 mL) was added Optiphase "MP" (2x10 mL). The radioactivity content was then determined using liquid scintillation counting. PAH and TEA accumulation in kidney slices are presented as a ratio of the radioactivity in the slice (100 mg wet weight) to that remaining in the incubation medium (0.1 mL): the S/M ratio.

The pyruvate content of the medium was assayed using the method of Bergmeyer /13/ involving L-lactic acid dehydrogenase catalysed conversion of pyruvate into lactate. This reaction is coupled to the oxidation of the reduced form of nicotinamide adenine dinucleotide and the results are compared with those of a previously constructed standard curve. The ammonia content was determined in a similar manner using glutamate dehydrogenase and 2-oxoglutarate /14/.

III. RESULTS AND DISCUSSION

Kidney slice methodology was chosen to investigate in detail the activity of a series of substituted S-phenyl-L-cysteine conjugates as rat renal C-S lyase inhibitors. This technique is an *in vitro* model which displays the complete physiology of the renal cortex, and the renal cortical slices used possess an organic cation and anion trans-basolateral membrane transport function.

All experiments were performed in an atmosphere of oxygen (100%) to prevent the onset of hypoxia and subsequent cell death. The first experiment involved determining the extent of C-S lyase activity present in the renal slice using NAcPCBC (3) as the substrate, assaying for the pyruvate and ammonia which were produced. Additionally, the effect of this cysteine conjugate on radiolabelled markers of the organic ion transport system: anionic, sodium [^{3}H]-*p*-amino-hippurate (PAH) and cationic, [^{14}C]-tetra-ethylammonium bromide (TEA) was determined.

In a 90 minute incubation ammonia production increased from 6.46 nmol/mg kidney slice (control endogenous ammonia) to 12.19 nmol/mg kidney slice in the presence of 0.5 mM NAcPCBC (Figure 4). Pyruvate was detected during a 3 hour incubation period, and the level of pyruvate was seen to double from control levels in the presence of 0.5 mM NAcPCBC: typically from 0.20 to 0.39 nmol/mg kidney slice, or from 0.99 to 1.80 nmol/mg kidney slice

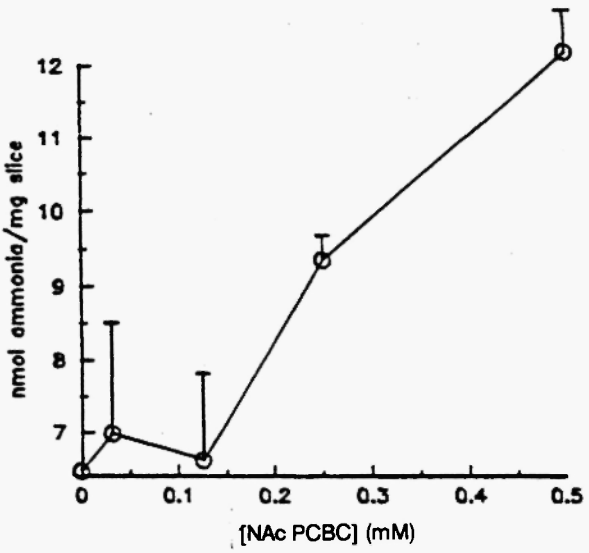

Fig. 4: The production of ammonia from NAcPCBC in rat renal cortical slices.

(mean values, n=6, from 0.34 to 0.75 nmol/mg kidney slice respectively).

An inspection of these results reveals that the deacetylation of NAcPCBC (3) and hence its conversion into (2), must occur prior to rat renal C-S lysis of the substrate resulting in ammonia and pyruvate production. Rat renal C-S lyase in kidney slices was inhibited (% inhibited from control values using 0.5 mM NAcPCBC as the substrate) by S-2,6-dinitrophenyl- (2,6-DNPC) (8) (88%), N-acetyl-S-2,6-dinitrophenyl-(NAc-2,6-DNPC) (11) (80%), N-acetyl-S-3,4-dinitrophenyl- (NAc-3,4-DNPC) (9) (70%), N-acetyl-2-chloro-4-nitrophenyl (NAc-2-Cl-4-NPC) (10) (48%), S-2-nitrophenyl- (2-NPC) (4) (42%), and S-4-nitrophenyl-L-cysteine (4-NPC) (5) (10%) when monitored for pyruvate production (Table 1) at 0.5 mM inhibitor concentration. Ammonia production was also monitored (Table 2) and the inhibition profile is as follows: 2,6-DNPC (100%), NAc-2,6-DNPC (86%), NAc-3,4-DNPC (94%), NAc-2-Cl-4-NPC (100%), S-2-NPC (70%), and S-4-NPC (62%).

The effect of NAcPCBC on PAH and TEA accumulation in rat kidney slices is shown in Figure 5 where the slice/medium ratio (S/M) for TEA decreases from 8.86 (control experiments) to 2.43 in

TABLE 1

The Effect of S-Phenyl Substituted L-Cysteine Conjugates on Pyruvate Production

[I] mM		0.00	0.0625	0.125	0.25	0.50
2,6-DNPC	(8)	0.251	0.144	0.243	0.177	0.031
NAc-2,6-DNPC	(11)	0.217	0.131	0.138	0.046	0.044
NAc-3,4-DNPC	(9)	0.187	0.183	n.d.	0.155	0.057
NAc-2-Cl-4-NPC	(10)	0.170	0.018	0.000	0.059	0.088
2-NPC	(4)	0.789	0.810	1.130	0.457	0.000
4-NPC	(5)	0.811	0.037	0.312	0.709	0.729

Pyruvate production is reported as nmol/mg kidney slice/incubation using 0.5 mM NAcPCBC as the substrate
n.d. = value not determined
[I] = Concentration of S-phenyl-L-cysteine conjugate

TABLE 2

The Effect of S-Phenyl Substituted L-Cysteine Conjugates on Ammonia Production

[I] mM		0.00	0.0625	0.125	0.25	0.50
2,6-DNPC	(8)	20.84	3.68	9.05	4.10	0.00
NAc-2,6-DNPC	(11)	5.03	1.27	2.68	0.70	0.73
NAc-3,4-DNPC	(9)	4.20	2.85	1.49	0.92	0.25
NAc-2-Cl-4-NPC	(10)	6.46	2.83	n.d.	2.61	0.00
2-NPC	(4)	4.83	6.81	n.d.	2.36	1.43
4-NPC	(5)	4.00	3.17	4.62	5.33	1.53

Ammonia production is reported as nmol/mg kidney slice/incubation using 0.5 mM NAcPCBC as the substrate
n.d. = value not determined
[I] = Concentration of S-phenyl-L-cysteine conjugate

the presence of 0.5mM NAcPCBC. Similarly, a reduction in the S/M ratio was observed for PAH, from 4.67 (control experiments) to 2.00. The percentage decrease in S/M ratio for TEA was 72.6, and that for PAH was 57.2. The accumulation of both markers of the

organic ion transport system (i.e. the S/M ratio of PAH and TEA) in the kidney slice are reduced and thus, NAcPCBC inhibits these transport mechanisms in kidney slices.

TABLE 3

The Effect of S-Phenyl Substituted L-Cysteine Conjugates on Organic Cation and Anion Transport

[conjugate] mM			0.0625	0.125	0.250	0.500
2,6-DNPC	(8)	^{3}H-PAH	43.6	39.0	44.5	39.0
		^{14}C-TEA	58.6	49.1	52.9	42.9
NAc-2,6-DNPC	(11)	^{3}H-PAH	36.8	41.2	44.3	48.6
		^{14}C-TEA	40.4	38.2	39.7	26.3
NAc-3,4-DNPC	(9)	^{3}H-PAH	38.5	25.9	28.5	41.0
		^{14}C-TEA	64.0	51.9	51.4	52.9
NAc-2-Cl-4-NPC	(10)	^{3}H-PAH	54.4	42.6	58.1	61.8
		^{14}C-TEA	79.3	80.1	86.6	88.8
2-NPC (HCl)	(4)	^{3}H-PAH	58.9	59.7	61.2	69.6
		^{14}C-TEA	65.7	56.7	54.2	57.3
4-NPC (HCl)	(5)	^{3}H-PAH	48.1	39.5	30.7	46.2
		^{14}C-TEA	56.7	56.1	57.4	51.5

The above data report the percentage decrease in slice/medium rat from control values which were determined in the absence of any cysteine conjugate

The nitrophenyl substituted cysteine conjugate C-S lyase inhibitors disrupted the uptake of PAH and TEA (Table 3). This latter characteristic was not inhibitor concentration dependent, neither was it on a comparable level to the inhibition achieved using NAcPCBC as the substrate for rat renal C-S lyase.

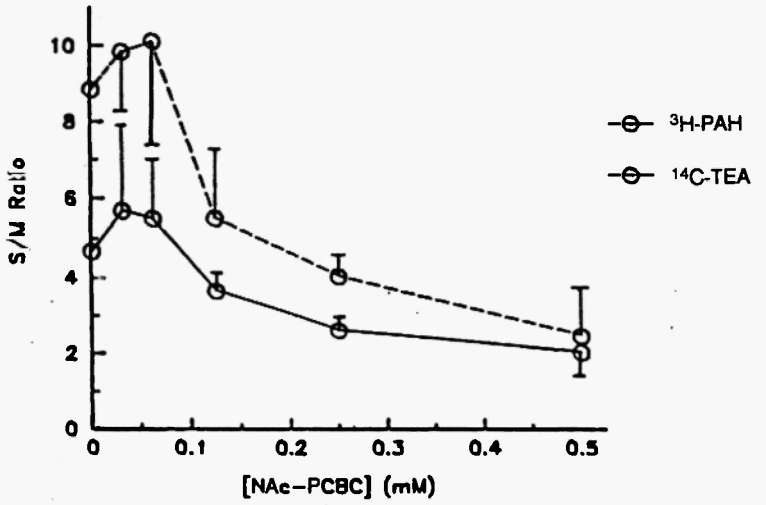

Fig. 5: The effect of NAcPCBC on PAH and TEA accumlation in rat renal cortical slices.

IV. CONCLUSIONS

It is evident from these studies that, in a step prior to the C-S lyase turnover of the substrate, the primary amine (2) (pentachlorobuta-1,3-dienyl-L-cysteine) must be liberated from NAcPCBC (3) by a deacetylase. Since the primary amine is a structural requirement for significant C-S lyase enzyme activity such an amine must bind at the active site of the pyridoxal phosphate dependent enzyme. (Aminooxyacetic acid and hydroxylamine bind to this cofactor resulting in essentially complete loss of enzyme activity).

The most effective inhibitor of rat renal C-S lyase in our kidney slice studies was 2,6-DNPC. Since NAc-2,6-DNPC is also an inhibitor of this enzyme in kidney slices, but not in crude kidney cytosol, a deacetylase enzyme is evidently present in the former system /15/. This result is supported by the activity of NAcPCBC as a substrate in the kidney slice preparation. Therefore, the nitrophenyl-L-cysteine conjugates display C-S lyase inhibition in two *in vitro* systems – in kidney cytosol and in the kidney slice model.

V. ACKNOWLEDGEMENTS

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