# 5α,6α-Epoxy-cholestan-3β-ol (cholesterol α-oxide): A specific substrate for rat liver glutathione transferase B

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A semi-micro assay was developed for the conjugation of 5α,6α-epoxy-cholestan-3β-ol (cholesterol α-oxide) with glutathione. The soluble supernatant of rat liver homogenate catalysed the reaction at a rate of 0.2–0.5 pmol.min$^{-1}$.mg protein$^{-1}$ with 4μM cholesterol α-oxide, while the reaction in the presence of GSH alone was barely detectable. Enzymic activity in the soluble supernatant was due equally to the two forms of glutathione transferase B (~100 pmol.min$^{-1}$.mg protein$^{-1}$), glutathione transferases AA, A, C and E being unreactive. The activity of purified glutathione transferase B was about 5-times that expected from the activity of the soluble supernatant. Complex enzyme kinetics were obtained suggestive of substrate inhibition.

*Glutathione transferase B* *Cholesterol α-oxide substrate*

## 1. INTRODUCTION

The glutathione transferases (EC 2.5.1.18) are a family of enzymes, widely distributed in nature, that catalyse the reaction of a variety of electrophiles with glutathione (GSH) [1–3]. They have numerous suggested functions generally related to detoxication or protective mechanisms including the intracellular transport of bilirubin and haem, the prevention of lipid peroxidation and the conjugation of a variety of reactive carcinogen and drug metabolites with GSH [3]. Almost all studies of GSH conjugation by these enzymes have been concerned with exogeneous compounds, but the recent discovery of the structure of leukotrienes [4,5] shows that GSH conjugation of reactive endogenous metabolites also occurs. Another possible endogenous reactant is 5α,6α-epoxy-cholestan-3β-ol (cholesterol α-oxide), a possible carcinogen [6,7] which is formed during microsomal lipid peroxidation [8]. This is converted to the GSH conjugate by the soluble supernatant of rat liver homogenate [9]. Here the rat liver GSH transferase responsible for the conjugation of cholesterol α-oxide is identified.

## 2. MATERIALS AND METHODS

[4-$^{14}$C]Cholesterol (58 mCi/mmol) was obtained from Amersham International (Bucks). USP-cholesterol from Sigma London Chemical Co. (Poole, Dorset) was recrystallised twice from ethanol–water. Cholestan-3β,5α,6β-triol was obtained from Steraloids (Croydon). Plastic-backed silica gel 60 TLC plates were from E. Merck (Darmstadt).

Unlabelled and $^{14}$C-labelled 5α,6α-epoxycholestan-3β-ol were synthesized using *m*-chloroperbenzoic acid [10] and purified by silica gel TLC with diethyl ether as solvent at 4°C [11]. It was stored in toluene at 4°C. [$^{14}$C]3β,5α-Dihydroxycholestan-6β-*S*-yl glutathione was synthesized and purified by TLC as in [9]. The compound was ninhydrin reactive and yielded a characteristic blue spot at 80°C after spraying with 50% $H_2SO_4$. The conjugate was stored in dry butanol at −20°C.

GSH transferases were purified from rat liver by a modification of the method in [12] in which isoelectric focusing was carried out between affinity chromatography and hydroxylapatite chromatography.

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Assays of the conjugation of cholesterol $\alpha$-oxide with GSH were carried out in 9 × 50 mm test tubes, which routinely contained 65 $\mu$l 0.1 M K-phosphate (pH 7.0) (± GSH transferases) and an appropriate amount of substrate added in 3 $\mu$l acetone. The tubes were shaken gently for 10 min at 30°C and the reaction initiated by addition of 10 $\mu$l phosphate buffer containing GSH. The reaction was stopped by the addition and rapid mixing of 6 $\mu$l 3 $N$ acetic acid together with 0.13 ml water-saturated $n$-butanol. After centrifugation (1000 × $g$, 10 min) a measured portion of the butanol extract (routinely 50 $\mu$l) was analysed by TLC on silica gel using ethyl acetate:acetic acid:$n$-butanol:$H_2O$ (6:3:2:2 v/v) as solvent [9]. After drying, the plate was run for twice the distance in diethyl ether at 4°C to separate cholestan-3$\beta$,5$\alpha$,6$\beta$-triol, a hydrolysis product, from unreacted cholesterol $\alpha$-oxide. The GSH conjugate, cholestan-3$\beta$,5$\alpha$,6$\beta$-triol and cholesterol $\alpha$-oxide from the reaction mixtures were identified by a comparison with standard compounds detected separately by charring with 50% $H_2SO_4$. The three components separated and identified as above were transferred to scintillation vials, the GSH conjugate was pretreated with 0.3 ml methanol:1 N NaOH (1:1), v/v), and radioactivity determined using 7 ml of scintillation fluid (10 g 2,5-diphenyloxazole; 1 g 1,4-di-2-(5-phenyloxazolyl)-benzene, 500 ml Triton-X-100 and 21 sulphur-free toluene).

## 3. RESULTS AND DISCUSSION

The soluble supernatant of rat liver homogenate catalysed the conjugation of GSH with cholesterol $\alpha$-oxide at 0.2–0.5 pmol.min$^{-1}$.mg protein$^{-1}$ (table 1) confirming the findings in [9] where an assay system was used which also contained 5% methanol and 0.025% Tween 80 to solubilise the substrate. Purification of the major rat liver GSH transferases demonstrated (table 1) that the activity present in the soluble fraction was due only to the two forms of GSH transferase B, a degree of specificity unusual among GSH transferases [13].

Since the activity is present in transferase B consisting of YaYa or YaYc subunits but not in transferase AA which consists of YcYc subunits [15], it seems to be associated with a Ya-type subunit. This is in accord with the presence of a high affinity hydrophobic binding site on Ya [16]

Table 1

GSH conjugation of cholesterol $\alpha$-oxide by rat liver GSH transferases (pmol.min$^{-1}$.mg protein$^{-1}$)

| Enzyme | Subunit composition[a] | [GSH] (mM) | Conjugation rate |
|---|---|---|---|
| Dialysed soluble supernatant | | 4 | 0.2–0.5 |
| Transferase B | YaYa | 4 | 60–110 |
| Transferase B | YaYc | 4 | 60–110 |
| Transferase AA | YcYc | 4 | (<0.2)[c] |
| Transferase E | [b] | 10 | (<0.2)[c] |
| Transferase A | YbYb | 4 | (<0.2)[c] |
| Transferase C | YbYb | 4 | (<0.2)[c] |

[a] Determined by SDS–polyacrylamide gel electrophoresis with nomenclature as in [14]

[b] The subunit composition of GSH transferase E does not correspond exactly with Ya, Yb or Yc

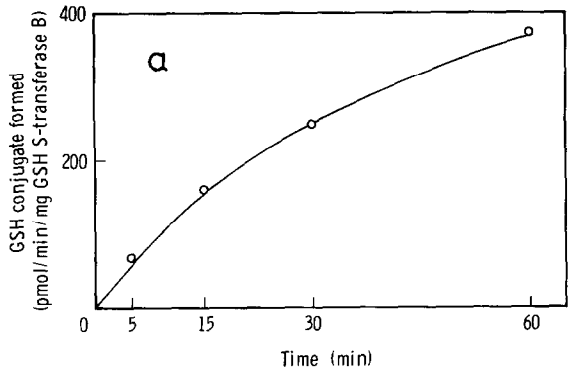
[c] Undetectable

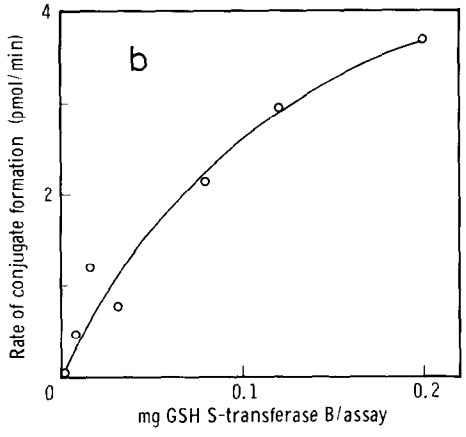
Assays were carried out as described in the text using a 10 min reaction period and 3–4 $\mu$M cholesterol $\alpha$-oxide (~$10^4$ dpm). Detectable rates are the range of 5 or more determinations with separate enzyme preparations

for which cholesterol is a ligand [17], and also with the specific $^5\Delta$-3-ketosteroid isomerase activity of transferase B. However, unlike steroid isomerase activity which is twice as high in YaYa as in YaYc [18], the ability to conjugate cholesterol $\alpha$-oxide did not differ significantly between the two forms of GSH transferase B, and was more comparable with their similar cholesterol-binding activities (unpublished).

Curiously, the activity of the purified enzymes was about 7-fold higher than that expected from the activity of the whole soluble fraction which contains up to 4% of transferase B. This suggests that the substrate may be partly unavailable to the transferases in the unfractionated sample either because the cholesterol $\alpha$-oxide is sequestered in lipoproteins or because endogenous ligands occupy the substrate binding site. No hydrolysis of the GSH conjugate to cholestan-3,5,6-triol was detected in the presence of transferases, the soluble supernatant, or the whole rat liver homogenate.

The time course and concentration dependence



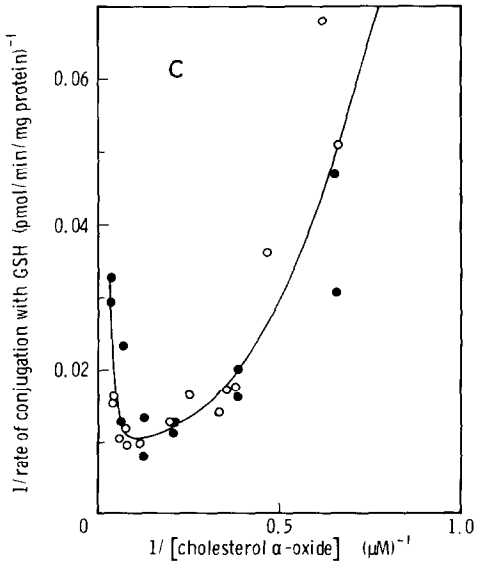




Fig. 1. Characterisation of the conjugation of cholesterol $\alpha$-oxide by GSH *S*-transferase B: (a) time course of reaction; (b) dependence upon protein concentration, assays contained 4$\mu$M cholesterol $\alpha$-oxide; (c) dependence on concentration of cholesterol $\alpha$-oxide, assays contained 25$\mu$g transferase B and were incubated with GSH for 10 min. The closed and open symbols represent experiments with two preparations of GSH transferase B. Assays were carried out as described in the text using a mixture of equal amounts of the two forms of GSH *S*-transferase B (no significant differences were obtained using either form by itself).

of the reaction of cholesterol $\alpha$-oxide with transferase B is shown in fig. 1. The kinetic pattern obtained (fig. 1c) is complex and suggests that a form of substrate inhibition may occur. This could be due to the binding of substrate in a catalytically inactive orientation such as is thought to occur with electrophilic metabolites of *N,N'*-dimethyl-4-aminoazobenzene [19,20].

The maximum rate obtainable (~100 pmol. $min^{-1}$. mg $protein^{-1}$) is low compared with commonly used exogenous substrates [13] but it represents a considerable stimulation of the non-catalytic rate which is barely detectable under these assay conditions yielding a second-order rate constant of $3 \times 10^{-11}$ l. $mol^{-1}$. $min^{-1}$.

Since cholesterol $\alpha$-oxide has been shown to be a significant product of microsomal lipid peroxidation [8] and occurs in human sera [21], it is possible that the transferase-catalysed conjugation with GSH constitutes a detoxication pathway in vivo.

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