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Hepatic and extra-hepatic glutathione-S-transferase activity in wild pigeons *(Columba livia)*

ASHWINI KUMAR, M. M. HUSAIN, HASAN MUKHTAR and
C. R. KRISHNA MURTI
Industrial Toxicology Research Centre, Post Box No. 80, Mahatma Gandhi Marg, Lucknow 226 001

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Abstract. Glutathione-S-transferase (EC 2.5.1.18) activity was assayed in hepatic and extra-hepatic tissues of pigeons using l-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene as substrates. Gluthathione-S-transferase activity towards l-chloro-2,4-dinitrobenzene in pigeon was in the order: kidney >liver >testes >brain >lung>heart. The enzyme activity with 1-chloro-2,4-dinitrobenzene as substrate was 40-44 times higher in pigeon liver and kidney than that observed with 1,2-dichloro-4-dinitrobenzene as substrate. K_m values of hepatic and renal glutathione transferase with l-chloro-2,4-dinitrobenzene as substrate were 2.5 and 3 mM respectively. Double reciprocal plots with varying reduced gluthathione concentrations resulted in biphasic curves with two K_m values (liver 0.31 mM and 4mM; kidney 0.36 mM and 1.3 mM). The enzyme activity was inhibited by oxidized gluthathione in a dose-dependent pattern. 3-Methylcholanthrene elicited about 50% induction of hepatic glutathione transferase activity whereas phenobarbital was ineffective.

Keywords. Avian glutathione-S-transferase; oxidized glutathione; 3-methylcholanthrene; phenobarbital; inhibition by oxidized glutathione; glutathione-S-transferase.

Introduction

Glutathione-S-transferases, a family of cytosolic enzymes with overlapping substrate specificities, play an important role in the biotransformation of many xenobiotics. Enzymic reactions involving conjugation of glutathione with a variety of electrophiles have been described (Habig *et al*., 1974; Hayakawa *et al*., 1975; Chasseaud, 1976; Mukhtar and Bresnick, 1976a; Jerina and Bend, 1977; Jakoby, 1978). S-conjugate formation by glutathione-S-transferases results in detoxification of a wide variety of electrophiles and is the first step in the syntheses of mercapturic acids (Chasseuad, 1976; Jakoby, 1978). Glutathione-S-transferase activity is widely distributed in mammals (Kaplowitz *et al*., 1976; Mukhtar and Bend, 1977; Mukhtar *et al*., 1978: Bend *et al*., 1979; Cantfort *et al*., 1979), aquatic organisms (James *et al*., 1979; Nimmo *et al*., 1979), insects (Motoyama *et al*, 1978), earthworm (Stenersen *et al*., 1979) and in plants (Guddewar and Dauterman, 1979).

Abbreviations used: Chloro-dinitrobenzene, l-chloro-2, 4-dinitrobenzene; dichlorodinitrobenzene 1,2-dichloro-4-nitrobehzene; GSH, gluthathione (reduced); GSSG, glutathione (oxidized); 3MC, 3-methylcholanthrene.

Birds are the first victims of exposure to toxic chemicals in the air and as such it was of interest to study the mechanism of xenobiotic biotransformation in birds. A preliminary report on the presence of glutathione-S-transferase activity in wild birds has appeared (Witt and Snell, 1968). To the best of of our knowledge no detailed systematic characterization of glutathione-S-transferase of avain species has been attempted so far. The present paper deals with the occurrence, localization and characterization of glutathione-S-transferase activity in wild pigeon (*Columba livia*).

Materials and methods

1,2-Dichloro-4-nitrobenzene (dichloronitrobenzene) and 1-chloro-2, 4-dinitrobenzene (chlorodinitrobenzene) were supplied by Eastman Organic Chemicals and the Aldrich Chemical Co., Milwaukee, Wisconsin, USA, respectively, and were recrystalized from ethanol prior to use. Gluthathione reduced (GSH) and glutathione oxidized (GSSG) were purchased from SISCO Research Laboratories, Bombay. 3-Methylholanthrene was a product of Sigma Chemical Co., St. Louis, Missouri, USA.

Animals

Male wild pigeons (200-250 g) were obtained with the help of commercial trappers. Albino rats (150-200 g) derived from the animal breeding colony of this Centre and raised on a commercial pellet diet (Hindustan Lever, Bombay) were used.

Subcellular fractionation:

Pigeons or albino rats were exsanguinated, cut open from abdomen, and the desired tissues were removed, blotted free of blood, washed twice in ice-cold 0.05 M phosphate buffer (pH 7.4), containing 0.15 M KCl. Tissue homogenates (25% w/v) were prepared inbuffered KCl using a Potter-Elvehjem glass homogenizer fitted with a teflon pestle. The homogenates were centrifuged at 3000 g for 15 min and the result ing supernatant was recentrifuged at 9000 *g* for 20 min to recover the postmitochondrial fraction. When needed the mitochondrial fraction was washed twice before use. Cytosolic fraction was obtained by centrifuging the post-mitochondrial supernatant at 105,000 *g* for 60 min in a Beckman ultracentrifuge. The resulting supernatant was carefully transferred to another tube and was kept at – 16°C till the enzyme activity was assayed. Storage upto a week did not decrease the enzyme activity.

Enzyme assay:

Glutathione transferase activity using chlorodinitrobenzene or dichloronitrobenzene as substrates was assayed spectrophotometrically essentially as described by Habig *et al*. (1971). The cuvettes (final volume of 3.0 ml) contained 0.1 M phosphate buffer (pH 6.5 for assaying chlorodinitrobenzene and pH 7.8 for dichloronitrobenzene activity), 1 mM GSH and 1 mM of either substrate and suitable aliquots (usually 20 μl) of appropriately diluted enzyme from the different sources. Change in absorbance at 340 nm (for chlorodinitrobenzene) and 344 nm (for dichloronitrobenzene) was followed against a blank containing all reactants excepting enzyme protein, Specific activity was expressed as nmol conjugate formed/min/mg protein

using a molar extinction coefficient of 9.6 and 8.5 for chlorodinitrobenzene and dichloronitrobenzene respectively. Protein was determined according to Lowry *et al*., (1951) using bovine serum albumin as standard.

Results

Glutathione-S-transferase activity towards chlorodinitrobenzene in kidney and liver cytosols of male pigeons was linear upto 160 μg of protein in the incubation mixture at pH 6.5. In subsequent experiments all the assays were done at this or lower protein concentrations. The effect of varying pH of incubation mixture on hepatic and renal cytosolic glutathione-S-transferase activity with chlorodinitrobenzene as substrate is given in table 1. It was seen that the highest specific activity

Table 1. Effect of pH on glutathione-Stransferase activity towards chlorodinitrobenzene as substrate using pigeon liver and kidney cytosols.

pH	nmol conjugate formed/ min/mg of protein	
	Liver	Kidney
5.5	100	140
6.0	251	491
6.5	552	632
7.0	750	843
7.5	1255	1250
8.0	1192	1545

The experiment was repeated three times and almost identical values were obtained. Data of one such experiment is given. All assays were done using phosphate buffer 0.1 M of desired pH value.

was observed at pH 7.5 and 8.0 for liver and kidney glutathione-S-transferase respecively. At higher pH values (7.0 or more) nonenzymatic rate was very high. A compromise was thus made and for all subsequent experiments pH 6.5 was used.

Localization of the enzyme in different sub-cellular fractions of pigeon liver is illustrated by the results summarized in table 2 indicating that almost all the activity is located in the particulate-free cytosol fraction. A relative enrichment of 60% in the specific activity in the cytosol fraction as compared to total homogenate was observed.

The relative specific activities of glutathione-S-transferase in different tissues of pigeon and their comparison with corresponding activities of rat tissues are given in table 3. It could be seen that the activity of enzyme in pigeon tissues was in the order: kidney > liver > testes > brain > lung > heart, whereas in rat a different pattern was observed and the activities were in the order: testes > liver > brain > kidney > heart > lung. Glutathione-S-transferase activity in kidney cytosols of

Table 2. Specific activity of glutathione-S-transferase in different subcellular fractions of pigeon liver

Fraction	Specific activity (nmol conjugate formed/ min/mg protein)
Homogenate	248
Nucleus	N.D.
Mitochondira	N.D.
Microsome	N.D.
105,000 g supernatant	391

Experiment was repeated 3 times and almost identical results were obtained. The data of one such experiment is given. Chloronitrobenzene was used as substrate.
N.D = not detectable.

Table 3. Glutathione-S-transferase activity with chlorodinitrobenzene as the substrate in pigeon and rat tissue cytosols

Tissue	Specific activity (nmol conjugate formed/ min/mg protein)	
	Pigeon	Rat
Kidney	786	237
Liver	391	1625
Testes	343	1666
Brain	178	379
Lung	123	120
Heart	101	188

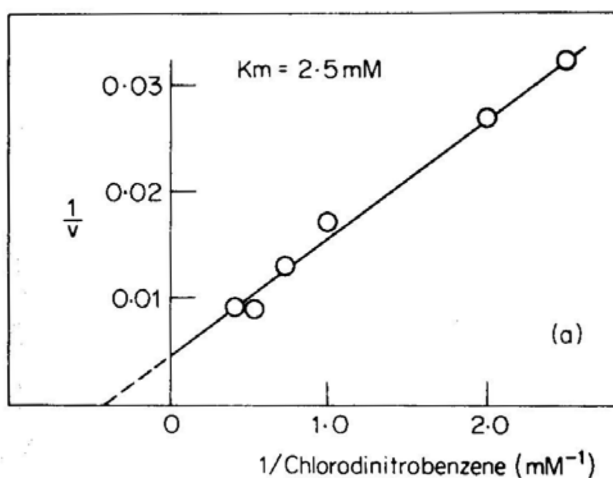
The values given are from a pooled tissue preparation from 4 animals.

pigeon was 3.3 times that of rat kidney, whereas pigeon liver had only 24% of the activity of rat liver. Glutathione-S-transferase activity with chlorodinitrobenzene as substrate in pigeon liver and kidney was 40-44 times higher than that observed with dichloronitrobenzene as substrate (table 4).

The apparent K_m values for chlorodinitrobenzene were about 2.5 and 3.0 mM for the liver and kidney transferases (figure 1a, b). Lineweaver-Burk plots for glutathione-S-transferase activity with varying glutathione concentrations gave a biphasic curve

Table 4. Comparison of glutathione-S-transferase activity in pigeon liver and kidney using chlorodinitrobenzene and dichloronitrobenzene.

Substrates	Specific activity (nmol conjugate formed/ min/mg protein)	
	Liver	Kidney
Chlorodinitrobenzene	597	800
Dichloronitrobenzene	15	18
Chlorodinitrobenzene/ dicnloronitrobenzene	40	44



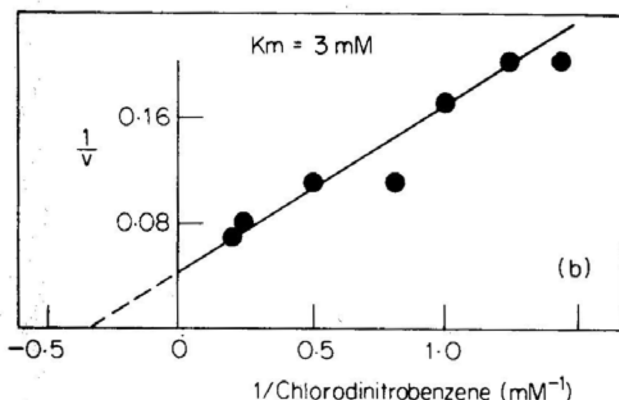


Figure l(a). Lineweaver-Burk plot showing the effect of varying concentrations of chlorodi-itrobenzene on the reaction rate of pigeon liver glutathione transferase. Assays were conducted using liver cytosol as the enzyme source (100 μ g protein). Concentration of the substrate (0.3 to 1 mM) was varied and that of glutathione-reduced (1 mM) was kept constant. Other conditions were as described in the text.

Figure l(b). Lineweaver-Burk plot showing the effect of chlorodinitrobenzene concentration on the reaction rate of pigeon kidney glutathione-S-transferase. Assays were conducted using kidney cytosol as enzyme source (100 μ g protein). Concentration of the substrate (0.5 to 2 mM) was varied and that of GSH (1 mM) was kept fixed. Other conditions were as described in text.

both for liver and kidney enzyme giving $2K_m$ values; liver 4.0,0.31 mM and kidney 1.3,0.36 mM (figure 2a, b). Pigeon liver glutathione-S-transferase activity was inhibited by GSSG in a dose-dependent pattern (figure 3). Figure 2a also indicates that GSSG was a noncompetitive inhibitor of pigeon hepatic glutathione-S-transferase as evident by change brought about in V_{max} value by GSSG.

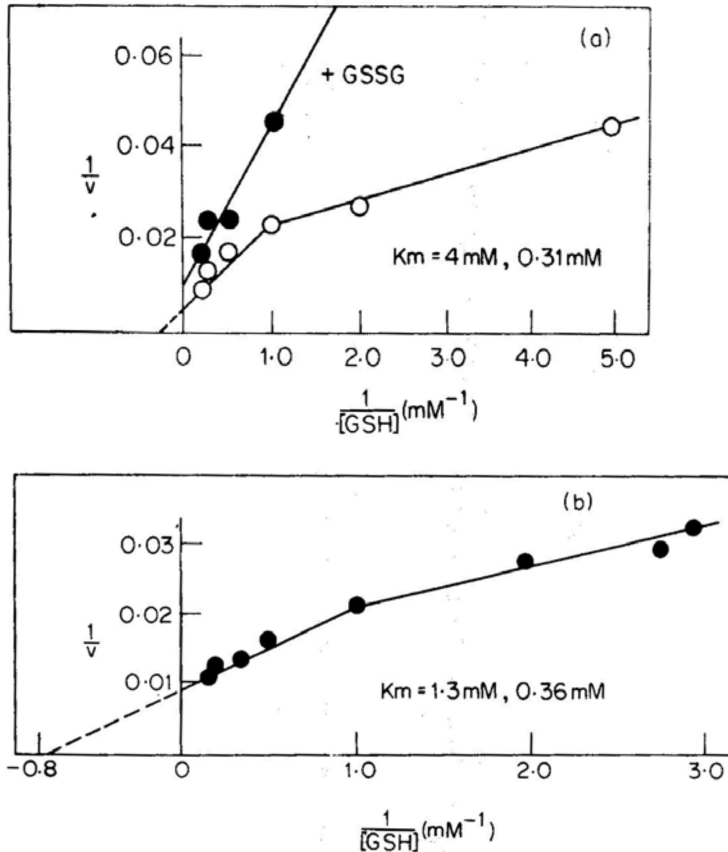


Figure 2(a). Lineweaver-Burk plot showing the effect of GSH concentration on the rate of the reaction catalyzed by pigeon liver glutathione-S-transferase. Enzyme activity was assayed using liver cytosol as the enzyme source (100 μg protein), in the presence of varying concentrations of GSH (0.2 to 1 mM) and fixed concentrations of chlorodinitrobenzene (1 mM). The velocity in the presence of varying concentrations of GSH and at a fixed concentration of GSSG (1 mM) as determined. GSH, O;GSH+GSSG, ●.

Figure 2(b). Lineweaver-Burk plot showing the effect of GSH concentration on the reaction rate of pigeon kidney glutathione transferase. Enzyme activity was assayed using kidney cytosol as the enzyme source (100 μ g protein), in the presence of varying concentrations of GSH (0.3 to 1 mM) and fixed concentrations of chlorodinitrobenzene (1 mM).

The effect of phenobarbital or 3-MC administration on hepatic glutathine-S-transferase with chlorodinitrobenzene as substrate is shown by results given in table 5. Phenobarbital at the dose and time period studied did not produce any effect on the levels of hepatic glutathione-S-transferase whereas 3MC stimulated the activity by 65%.

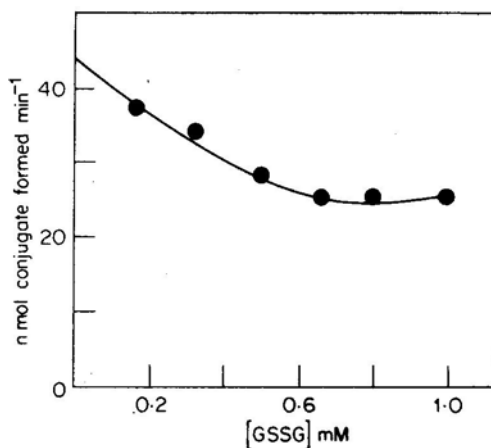


Figure 3. Effect of GSSG concentration on the reaction catalyzed by the pigeon liver glutathione-S-transferase. Varying amounts of GSSG (0.1 to 1 mM) were pre-incubated for 10 min at room temperature with the components of the reaction mixture (100 μ g enzyme protein). The enzyme activity was inhibited by the addition of chlorodinitrobenzene and was assayed as described in the text.

Table 5. Effect of 3-methylcholanthrene and phenobarbital administration to pigeon on hepatic glutathione-S-transferase activity.

Treatment	Specific activity (nmol conjugate formed/ min/mg protein)
Saline	473±147 (4)
Phenobarbital (72 h)	442±129 (4)
Peanut oil	585±163 (4)
3-Methylcholanthrene (48 h)	967±199[a] (3)

Chlorodinitrobenzene was used as substrate to measure the activity. The data represent mean ±S. D. of number of values given in paranthesis. Pigeons were injected intraperitoneally with phenobarbital (40 mg/kg body weight in saline on 3 consecutive days) or with 3-methylcholanthrene (40 mg/kg body weight, in peanut oil for 2 consecutive days). The pigeons were sacrificed 24 h after they had received the last treatment. Control animals received identical volume of saline or peanut oil. All injections or sacrifice were done between 10 and 11 A.M. to avoid any diurnal variation.
[a] $p<0.05$.

Discussion

Avian species are the major targets of attack, by air pollutants. Wild species of birds are equipped with cytochrome P-450 dependent mixed function oxidase system (Runnels and Khan, 1975; Sifri *et al*., 1975). Earlier studies from this laboratory revealed that tissues of wild pigeon possess considerable activity (as compared to rats) of drug metabolising enzymes and are, therefore, equipped for disposal of xenobiotics (Husain *et al*., 1979). The present studies demonstrate that, in addition to the mixed function oxidase system, wild pigeons are also equipped with cytosolic glutathione-S-transferases. The presence of significant amounts of glutathione transferase activities in hepatic and extra-hepatic tissues of pigeons is similar to the pattern of enzyme profile in rodent tissues (Bend *et al*., 1979) suggesting that most target tissues of pigeons are equipped to deal with the toxic effects of circulating electrophiles. In the pattern of localization of glutathione-S-transferase in tissue fractions, the manifestation of a biphasic Lineweaver-Burk plot with GSH and inhibition of enzyme activity by GSSG, the pigeon enzyme shows similarities to the rodent enzyme. Unlike the rodents, pigeons have, however, the highest activity in renal tissue. The enzyme in the kidney glutathione transferase is implicated in the transport of organic anions besides its role in the disposal of xenobiotics as mercapturic acid (Pegg and Hook, 1976). A high enzyme activity in renal tissue of pigeons may also be related to the operation of an overall excretary mechanism different from that of mammals.

Administration of 3-MC to pigeons resulted in the induction of hepatic enzyme activity whereas phenobarbital was ineffective. All the mammalian species investigated so far have shown response to pheno-barbital as inducer of hepatic enzyme activity whereas 3-MC is an inducer in only a few mammalian species. Thus Mukhtar and Bresnick (1976) reported that, in case ofrat liver 3MC and phenobarbital are inducers of enzyme activity for most substrates investigated, whereas for mouse liver 3MC was ineffective (Mukhtar and Bresnick, 1976b). Since the molecular mechanism of induction of the enzyme by selective inducers has not been elucidated, it is difficult to ascertain at this stage, the cause of such an unusual induction pattern of the enzyme activity in pigeons.

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