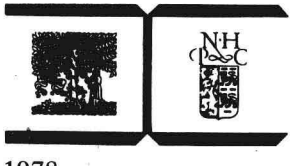
Conjugation Reactions in Drug Biotransformation

A. Aitio Editor

CONJUGATION REACTIONS IN DRUG BIOTRANSFORMATION

Proceedings of the Symposium on Conjugation Reactions in Drug Biotransformation held in Turku, Finland, July 23-26, 1978.

Editor
A. Aitio



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FOREWORD

Many biologically active chemicals lack activity per se; they become toxicologically or pharmacologically active through a variety of drug biotransformation reactions - sometimes these give rise to mutagenic or carcinogenic products. The potency of such a reactive intermediate depends on the balance between their formation and destruction. The formation is in many cases catalyzed by various mono-oxygenation enzymes, the destruction by conjugation enzymes. Thus the conjugation reactions play an important role by influencing the toxicity of many chemicals.

Conjugation of a chemical in the body usually increases the water solubility, which has profound effects on the disposition of the drug; in fact, conjugation is a prerequisite for the excretion of a variety of drugs. Conjugates have also been implicated as transport forms in the body. Transport of eg. dehydroepiandrosterone as a sulfate has long been recognized, but recent evidence suggests that glycosides also may be involved in the transport of steroid molecules both within the cell and through the cell membrane. Arylamine derivatives have been suggested to reach the urinary bladder as glucuronides, exerting their carcinogenic action there.

Recently it has been found that conjugation reactions do not always cause a decrease in the toxicity of compounds. Certain sulfate, glucuronide and glutathione conjugation reactions as well as acetylation have been implicated in the very formation of the active species, i.e. in toxication reactions.

The present book contains the invited lectures and abstracts of the poster demonstrations presented at the satellite symposium of the 7th IUPHAR Congress, named 'Conjugation Reactions in Drug Biotransformation', and held in Turku, Finland, July 23-26, 1978. Also included in the book, although substantially condensed, are the discussions. The main emphasis of the symposium was to elucidate the state of art of the conjugation reactions in toxication, detoxication, and disposition of drugs, as discussed above. Because of the limited time some conjugation reactions had to be excluded. This was done mainly in order to ensure a concentrated, and hopefully fruitful, three days of lectures and discussions for the participants.

My sincere thanks are due to the members of the scientific committee, consisting of Kaarlo Hartiala, Turku, John Bend, Research Triangle Park, Geoffrey Dutton, Dundee, Osmo Hänninen, Kuopio, Sten Orrenius, Stockholm, Pekka Uotila, Turku and Harri Vainio, Helsinki, as well as to all lecturers and participants.

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Turku, September 1978

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ALKENE AND ARENE OXIDE METABOLISM IN HEPATIC AND EXTRAHEPATIC TISSUES -- PHARMACOLOGICAL AND TOXICOLOGICAL ASPECTS.

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ABSTRACT

Adult rat serum contained glutathione *S*-transferase activity with styrene oxide and benzo(a)pyrene 4,5-oxide as substrates, which was 60% higher in males than in females. Rat testis, ovary, and adrenal had relatively high glutathione *S*-transferase activities (50-115% female hepatic values) and relatively low, but easily measured, epoxide hydrase activities (5-15% female hepatic values) with the epoxide substrates studied. Cytosolic and microsomal fractions of spermatogenic cells from rat testis had about twice the glutathione *S*-transferase and epoxide hydrase activities, respectively, of similar fractions from interstitial cells. Rat sperm also contained glutathione *S*-transferase, but not epoxide hydrase, activity. During pregnancy there was a significant increase in ovarian glutathione *S*-transferase activity, particularly with styrene oxide as substrate. Elevated enzyme activities persisted for 30 days after the cessation of lactation.

Benzo(a)pyrene 4,5-oxide was rapidly metabolized by the isolated perfused rat liver. Both epoxide hydrase and the glutathione *S*-transferases were major pathways. The glutathione conjugate was preferentially excreted into bile. There was little covalent interaction of the arene oxide with hepatic DNA or RNA.

INTRODUCTION

The microsomal cytochrome P-450-dependent monooxygenase system, which is present in liver and several extrahepatic tissues of vertebrate species, metabolizes many endogenous and exogenous compounds including drugs and environmental pollutants. Biotransformation by this enzymatic pathway can convert xenobiotics to less toxic (detoxication) or more toxic (metabolic activation) products. Olefinic and aromatic hydrocarbons are generally converted to alkene and arene oxides, respectively, during oxidative metabolism by this cytochrome P-450-dependent system[1]. These electrophilic epoxides, which are implicated as at least one chemical species responsible for the toxic effects of various hydrocarbons[2], are further biotransformed by microsomal epoxide hydrase (EC 4.2.1.63)[3]

diols or *trans*-dihydrodiols and by conjugation with glutathione, a reaction catalyzed by a family of cytosolic enzymes called the glutathione *S*-transferases (EC 4.4.1.7)[4,5]. Both epoxide hydrase and the glutathione *S*-transferases are present in many extrahepatic tissues of vertebrate and invertebrate species[5,6,7].

For many epoxides, hydration and conjugation with glutathione represent detoxication reactions. However, in the case of benzo(a)pyrene, the formation of benzo(a)pyrene 7,8-dihydrodiol from the corresponding arene oxide can be termed an activation step[8] since the dihydrodiol is the metabolic precursor of the isomeric benzo(a)pyrene 7,8-dihydrodiol-9,10-epoxides, which are potent mutagens[9]. Similar metabolic pathways have been reported for other polycyclic hydrocarbons. Thus, the cytochrome P-450-containing monooxygenase system, epoxide hydrase, and the glutathione *S*-transferases are involved in the metabolic activation and detoxication of many carcinogenic, mutagenic, and cytotoxic hydrocarbons. The quantitative relationships between these activation and detoxication pathways may be at least partially responsible for determining the target organ toxicity mediated by certain environmental or industrial pollutants.

Consequently, our laboratory is interested in the formation and subsequent metabolism of alkene and arene oxides by hepatic and extrahepatic tissues. In this report we describe the *in vitro* metabolism of an alkene and arene oxide by steroidogenic tissues, sperm, and serum of the rat and the biotransformation of benzo(a)pyrene 4,5-oxide by the isolated perfused rat liver.

MATERIALS AND METHODS

Enzyme Assays

Glutathione *S*-transferase activity of serum, sperm, or 176,000x*g* supernatant fraction of various tissues was assayed with ^{14}C-styrene oxide (SO, >99% radiochemical purity), ^{3}H-benzo(a)pyrene 4,5-oxide (BPO, >99% radiochemical purity), 1,2-dichloro-4-nitrobenzene (DCNB), or 2,4-dinitrochlorobenzene (DNCB) as described earlier[10,11]. Microsomal epoxide hydrase activity was assayed with SO or BPO as substrate, using the thin-layer chromatographic (TLC) assay of Jerina *et al.*[12]. Cytochrome P-450 content of hepatic, testicular, ovarian, and adrenal microsomes was determined by the method of Omura and Sato[13]. Microsomal benzo(a)pyrene hydroxylase (AHH) activity was determined basically as reported by Dehnen *et al.*[14,15].

Animals

Adult male or female Sprague-Dawley CD rats were used throughout.

Tissue Serum and Sperm Preparations

Tissues (testis, ovary, liver, or adrenal) and isolated interstitial or spermatogenic cells were homogenized in 4 volumes of ice-cold 0.15 M KCl/0.02 M HEPES buffer, pH 7.4, in a glass homogenizer fitted with a motor-driven Teflon pestle. Microsomes and microsomal supernatant fractions were obtained by differential centrifugation of tissue homogenates as previously described[10]. The protein content of subcellular fractions was determined by the method of Lowry *et al.*[16], with bovine serum albumin as the standard.

Serum was separated from rat blood by centrifugation at 600x*g* for 10 min (after standing at room temperature for 20 min).

The detailed procedures used for the preparation of interstitial cells and spermatogenic cells from rat testis have been described elsewhere[15]. Sperm was gently flushed from the vas deferens of rats by washing with ice-cold 0.1 M phosphate-buffered saline solution (PBS, pH 7.2). Collected sperms were washed twice with PBS by centrifugation at 100x*g* for 15 min (4°C). The small amount of red blood cell contamination was removed by submitting the sperm preparation to hypotonic shock for 20 sec in distilled water. After immediate readjustment to 300 mOs with 0.45 M NaCl, the sperm preparation was washed by resuspension (three times) and sedimentation at 1500x*g* for 15 min to remove cellular contamination. Finally the sperms were resuspended in PBS containing 0.1% cetyltrimethylammonium bromide and they were sonicated for 15 sec. Sonicated sperm suspensions were used as the enzyme source.

Perfused Liver

Livers from male rats (200 ± 20 g) were cannulated at the portal vein and the bile duct and were perfused *in vitro* with Krebs-Ringer bicarbonate buffer (130 ml, pH 7.4) containing 4.5% bovine serum albumin and 5 mM glucose[17]. ^{3}H-BPO (in 200 µl DMSO) was infused into the portal vein over a period of several minutes. The perfusion medium was sampled and the bile collected at various time intervals for analysis of BPO and its metabolites. Metabolites extracted from the perfusion medium and homogenized liver (ethyl acetate:acetone 2:1) were characterized by TLC (silica gel, 250 µm) developed in benzene:ethanol (19:1) or hexane:benzene:ethanol (20:2:1) and by high pressure liquid chromatography (HPLC, DuPont model 830) using a reverse phase ODS column eluted with a methanol:water gradient.

Water-soluble metabolites in bile, the perfusion medium and from homogenized liver were characterized by two-dimensional TLC. The silica gel plates were developed in the first direction with *n*-butanol:ethanol:water:conc. ammonium

hydroxide (4:1:1:1) and in the second direction with *n*-butanol:water:glacial acetic acid 4:1:1), both before and after enzymatic hydrolysis with sulfatase (type H-2, Sigma) or β-glucuronidase (bovine liver, Sigma). Metabolites were visualized under 360 nm UV light, by spraying with ninhydrin or fluorescamine and by spraying with a reagent for divalent sulfur[18].

Covalent binding of BPO-derived radioactivity to protein, RNA, and DNA was determined by published procedures[19,20].

RESULTS AND DISCUSSION

Rat serum contained glutathione *S*-transferase activity with the model alkene (SO) and arene oxide (BPO) substrates tested (Table 1) although the specific activity was only about 1% hepatic cytosolic activity in each case. In contrast, neither serum nor blood cells contained detectable epoxide hydrase activity with SO or BPO. Serum from male rats had higher glutathione *S*-transferase activity (about 60%) than that from females, reflecting the sex difference in enzyme activity of adult rat liver, which is not found in other tissues studied[21]. The fact that the developmental patterns of serum and hepatic glutathione *S*-transferase activity were virtually identical in the rat[11] further suggested that the serum activity was due to a release of enzyme from liver.

TABLE 1

GLUTATHIONE *S*-TRANSFERASE ACTIVITY IN SERUM OF MALE AND FEMALE RATS[a]

		GLUTATHIONE *S*-TRANSFERASE ACTIVITY	
SEX	SUBSTRATE	NMOL/MIN/ML SERUM	NMOL/MIN/MG PROTEIN
Female	Benzo(a)pyrene 4,5-oxide	3.16 ± 0.84[b]	0.052 ± 0.014
Male	Benzo(a)pyrene 4,5-oxide	5.02 ± 1.22	0.081 ± 0.018
Female	Styrene oxide	62.5 ± 9.4	0.98 ± 0.24
Male	Styrene oxide	99.8 ± 15.2	1.56 ± 0.42

[a]Data from reference 11.

[b]Mean ± SD, N = 4.

Although rat blood cells also contained glutathione *S*-transferases, specific blood cell activity (nmol/min/mg protein) was only about 50% that of plasma[11].

Not surprisingly, administration of the hepatotoxin carbon tetrachloride (1 ml/kg) caused a dramatic increase (11-fold) in serum glutathione *S*-transferase activity 24 hr after dosing, with a concomitant decrease in hepatic enzyme

activity[11]. It is interesting to speculate that this release of the cytosolic glutathione *S*-transferases, and the tripeptide glutathione, from damaged hepatocytes may protect other organs from toxicity mediated by circulating electrophiles.

The release of substantial amounts of the glutathione *S*-transferases and other cytosolic enzymes into blood appears to be diagnostic of tissue damage. Since the transferases are so widely distributed (e.g., lung, liver, kidney, intestine, testis, ovary, adrenal, brain, pancreas), there are very sensitive procedures for assaying activity, and as there are several different glutathione *S*-transferases[4] it may eventually be possible to detect chemical induced toxicity in extrahepatic organs should specific glutathione *S*-transferase isozymes be present there, which are released into serum as a result of cellular damage.

There is currently much concern over the mutagenic properties of certain chemicals that occur as industrial and environmental pollutants. Consequently, the ability of tissues in which germ cells develop, and of germ cells themselves, to metabolize xenobiotics is of interest. Steroidogenic tissues are also important for the synthesis and regulation of hormones and any interference in these processes by toxic exogenous chemicals could have a profound effect on animal homeostasis. For these reasons we have studied alkene and arene oxide biotransformation and mixed-function oxidation in testis, ovary, and adrenal of the rat (Table 2). Microsomal supernatant fractions from these tissues had high glutathione *S*-transferase activities with both substrates tested (50-115% specific female liver cytosolic values). Although microsomal epoxide hydrase activity was also present in each tissue, specific activities were much lower than in liver (only 5-15%). The data clearly indicate a substantial ability of these organs to detoxify electrophiles, particularly by conjugation with glutathione.

The glutathione *S*-transferases are known to be involved in the noncovalent binding and transport of endogenous molecules, such as cortisol metabolites[22], as well as in the catalytic transfer of glutathione to electrophilic substrates. It seems likely that the high concentration of the glutathione *S*-transferases present in these steroidogenic tissues evolved in response to the former role, or to conjugation of endogenous metabolites with glutathione, rather than as a protective mechanism against xenobiotics.

Microsomal AHH activity of testis and ovary was very low, relative to hepatic activities (0.2-0.4%) and these lower enzyme activities were reflected in the substantially lower microsomal cytochrome P-450 contents (4-17%) of ovary and testis than in liver. This data (Table 2) demonstrated that testis, ovary, and adrenal have all of the requisite enzymes for metabolic activation of xenobiotics such as the polycyclic aromatic hydrocarbons to mutagenic and carcinogenic

TABLE 2

CYTOSOLIC GLUTATHIONE *S*-TRANSFERASE, MICROSOMAL EPOXIDE HYDRASE AND BENZO(a)PYRENE HYDROXYLASE (AHH) ACTIVITIES AND CYTOCHROME P-450 CONTENT OF TESTIS, OVARY, ADRENAL, AND LIVER OF THE RAT[a]

PARAMETER	SUBSTRATE	SPECIFIC ACTIVITY OR CONTENT			
		TESTIS	OVARY	ADRENAL	LIVER[b]
Glutathione *S*-Transferase Activity[c]	BPO[d]	20.0 ± 1.1 (8)[e]	12.6 ± 0.9 (4)[f]	5.8 ± 1.4 (3)[f]	24.6 ± 3.2 (4)
	SO[g]	105 ± 3 (8)	95 ± 4 (4)	140 ± 5	122 ± 11 (3)
Epoxide Hydrase Activity[c]	BPO	0.8 ± 0.1 (8)	0.60 ± 0.47 (3)	0.39 ± 0.08 (3)	10.3 ± 1.8 (4)
	SO	1.6 ± 0.2 (8)	1.5 ± 0.3 (4)	1.1 ± 0.2 (3)	10.5 ± 1.32 (4)
AHH Activity[h]		5.2 ± 0.6 (4)	3.2 ± 0.3 (4)	----	1,560 ± 120 (3)
Cytochrome P-450 Content[i]		0.13 ± 0.02 (4)	0.03 ± 0.01 (5)	0.76 ± 0.13 (3)	0.77 ± 0.25 (4)

[a] Data from references 15, 23, 24, 25.

[b] From female rats.

[c] Nmol product formed/min/mg protein.

[d] Benzo(a)pyrene 4,5-oxide.

[e] Mean ± SD (N).

[f] Ovaries and adrenals of 20 rats were pooled for each determination.

[g] Styrene oxide.

[h] Pmol 3-hydroxybenzo(a)pyrene/min/mg protein.

[i] Nmol/mg microsomal protein.