specificity of transferases from different species is not conserved with respect to xenobiotics or artificial substrates (Lee et al., 1981). One of the known examples is lactate dehydrogenase from different species. The purified form of this enzyme exhibits quite similar specific activity among different species, when L-lactate and the natural coenzyme, NAD+, are used for the enzyme assay. However, when the coenzyme analogue thionicotinamide–adenine dinucleotide is used as the coenzyme for activity assay, great species variations of enzyme activity are observed (Anderson, 1982). Therefore, from the enzymological point of view, it is apparent that enzyme activities are conserved through the species evolution only for their natural substrates, but not for xenobiotics (Lee et al., 1981). In view of this consideration, it remains an open question regarding the natural substrates of glutathione S-transferases and their additional biological functions in vivo. Since the artificial electronophiles were used for the initial identification of this detoxification enzyme system, one would expect great species variations in terms of their multiple forms and their specificity to artificial substrates. Such a commonly observed phenomenon may become an important concern in choosing a proper animal species for the evaluation of biochemical transformation and toxicity (or carcinogenesis) of numerous xenobiotics (Lee et al., 1984). Although there are variations on the theme when applied to certain reactive amines (Lotlikar et al., 1966), it is now clear that the major source of the sulphur is glutathione, with the methyl group being donated by S-adenosine- l-methionine (AdoMet). Despite the isolation of several such compounds bearing a thiomethyl group, and indications of probable intermediates (Bhattacharya & Schultz, 1967; Chatfield & Hunter, 1973), a clear demonstration of the pathway that is followed resulted from an investigation of the metabolism of bromazepam to its thiomethyl derivative (Tateishi & Shimizu, 1976) and the subsequent demonstration of cysteine conjugate β-lyase activity by Tatemichi et al. (1978).

The thiomethyl group arises as the result of initial thioether formation from reactive electrophiles interacting with the tripeptide, glutathione, a reaction catalysed by the glutathione S-transferases (Jackoby, 1978). It will be recalled that the subsequent reactions leading to a mercapturic acid require the successive participation of γ-glutamyltranspeptidase, which removes glutamate, and a dipeptidase, which removes glycine (Tate, 1980). The resultant product is a thioether of cysteine (I) that undergoes acetylation in an acetyl-CoA(CoASAc)-linked reaction catalysed by cysteine thioether N-acetyltransferase (Duffel & Jackoby, 1982) to yield a mercapturic acid (II); deacetylation activity can lead to recycling of the mercapturate back to the conjugate (Suzuki & Tateishi, 1981), to form (I). The novel aspect shown in the scheme below, is a shunt pathway which appears to direct metabolism of a number of compounds away from mercapturic acid synthesis. Rather than undergoing acetylation, the thioether of cysteine is

**Cysteine conjugate β-lyase and the thiomethyl shunt**

WILLIAM B. JAKOBY and JAMES STEVENS

*Section on Enzymes and Cellular Biochemistry, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20205, U.S.A.*

Administration of one of a number of xenobiotics that do not contain sulphur leads to metabolic products that bear the –SCH3 group (reviewed by Tateishi & Shimizu, 1980; Jakoby et al., 1984). Although there are ‘variations on the theme’ when applied to certain reactive amines (Lotlikar et al., 1966), it is now clear that the major source of the sulphur is glutathione, with the methyl group being donated by S-adenosine-l-methionine (AdoMet). Despite the isolation of several such compounds bearing a thiomethyl group, and indications of probable intermediates (Bhattacharya & Schultz, 1967; Chatfield & Hunter, 1973), a clear demonstration of the pathway that is followed resulted from an investigation of the metabolism of bromazepam to its thiomethyl derivative (Tateishi & Shimizu, 1976) and the
cleaved by cysteine conjugate β-lyase (Tateishi et al., 1978; Stevens & Jakoby, 1983) to form a free thiol (III), whereas the alanine moiety is degraded to pyruvate and ammonia. The thiomethyl group is completed (IV) by action of thiol methyltransferase (Weisiger & Jakoby, 1979):

(I)  $$\text{RSCH}_2\text{CH(NH}_3\text{)}_2\text{CO}_2\text{H} \rightarrow \text{RSCH}_2\text{CH(NH}_3\text{)}_2\text{CO}_2\text{H}$$

(II)  $$\text{RSCH}_2\text{CH(NH}_3\text{)}_2\text{CO}_2\text{H}$$

(III)  $$\text{RSH} \rightarrow \text{RSCH}_2\text{CH}_2\text{NH}_3\text{CO}_2\text{H}$$

Interest in the key enzyme of the shunt, cysteine conjugate β-lyase, has led us to purify it, redefine its substrate specificity, and make certain assumptions as to its mechanism. The enzyme from rat liver is readily obtained in soluble form (Tateishi et al., 1978) and was purified by relatively normal methods to homogeneity by the criterion of gel electrophoresis (Stevens & Jakoby, 1983).

Cysteine conjugate β-lyase

The reaction catalysed by the β-lyase yields a free thiol, ammonia and pyruvate. Since pyruvate formation is easily measured in a continuous spectrophotometric assay with NAD and lactate dehydrogenase, a variety of possible substrates could readily be evaluated (Stevens & Jakoby, 1983). The enzyme is a dimer ($M_m = 47,000$ and $39,000$) for which an $M_n$ of about 100,000 has been estimated (Stevens & Jakoby, 1983).

With an alternative chromatographic assay procedure and partially purified enzyme, Tateishi et al. (1978) were able to show that S-4-bromophenyl-L-cysteine, S-2,4-dinitrophenyl-L-cysteine and S-4-nitrobenzyl-L-cysteine were substrates, whereas S-2,4-dinitrophenyl-d-cysteine and mercapturic acids were not. By use of the more general assay procedure, or with a fluorometric assay for kynurenine and its derivatives (Jakoby & Bonner, 1953), we have observed a broader spectrum of substrates that can be accommodated (Stevens & Jakoby, 1983). Thus aromatic derivatives such as those listed above, as well as S-2-benzoxazolyl-L-cysteine and S-3-hydroxy-L-kynurenine qualify as substrates, but so does the alkyl derivative S-1,2-dichlorovinyl-L-cysteine, as well as β-chloroalanine (Stevens & Jakoby, 1983; Jakoby et al., 1984).

Cystathionine, a number of other amino acids, and the methyl, ethyl, 2-chloroethyl, butyl, benzyl and 4-nitrophenylthioesters of L-cysteine, were all inactive as substrates.

Several indications that include inhibition by carbonyl reagents and by cysteine, as well as stabilization of the enzyme by pyridoxal phosphate (Stevens & Jakoby, 1983), suggest that this coenzyme participates in the reaction. Indeed, two cystathioninases (Flavin, 1975) and a bacterial lyase (Nomura et al., 1963), all catalysing formally analogous, if distinctively specific, reactions also involve pyridoxal phosphate, as does kynureninase, the last yielding alanine rather than pyruvate as a product (Jakoby & Bonner, 1953). On the basis of the participation of pyridoxal phosphate, the reaction with benzothiazolocysteine may be considered as an α,β-elimination that successively involves Schiff-base formation with the amino acid, discharge of the leaving group to yield an eamine, regeneration of active enzyme and a pyruvate imine, and the formation of pyruvate and ammonia (Stevens & Jakoby, 1983), although additional work is necessary to prove the mechanism. The suggestion is that of a Michaelis addition of a protein nucleophile to the β-carbon of the intermediate common to all of these reactions, the eamine. No matter which substrate is used, the reactive species inactivates the β-lyase after an average of about 600 catalytic cycles.

The thiomethyl shunt

If the enzymes of detoxication (Jakoby, 1982) function by preparing xenobiotics for excretion by making them more water-soluble and by blunting their pharmacological activity, it is strange that a major system of detoxication, that of mercapturic acid synthesis, be shunted away from this task. Instead, the shunt pathway takes a generally pharmacologically inactive product, well on its way toward excretion, and processes it to form a member of a toxic group of compounds, a thiol, that is then methylated to produce a less-water-soluble derivative. We do not have the answer to this apparent paradox.

It is clear, however, that the shunt pathway has the potential for toxicity, as witnessed by the reactivity of sulphur-containing cleavage products of the β-lyase. The extensive renal damage and aplastic anaemia, for example, first noted as the result of accidental feeding of cattle with trichloroethylene, has been elegantly analysed as being due to its metabolism to S-1,2-dichlorovinyl-L-cysteine, which subsequently produces a sulphur-bearing alkylation fragment that reacts covalently with tissue components (McKinney et al., 1957; Bhattacharya & Schultze, 1967); it should not go unnoticed that the dichlorovinylcysteine is an excellent substrate for the β-lyase from rat liver (Stevens & Jakoby, 1983).

A problem that can be evaluated more readily is the source of the enzyme or enzymes active responsible for the formation of the thiomethyl group. For this purpose, the use of 'germ-free' animals or animals treated in a manner aimed at greatly diminishing the gut flora, is a necessity. In the rat, both 2-acetamido-4-chloromethylthiazole (Bakke et al., 1981) and propachlor (Larsen & Bakke, 1981) yield thiomethyl derivatives, but both have been shown to arise because of action of the intestinal flora rather than due to action of mammalian enzymes. Bacteria appear to have the appropriate β-lyase (Saari & Schultze, 1965), but not the S-methylating enzyme (Weisiger et al., 1980), the latter representing a function of the intestinal wall or liver. Nevertheless, a number of compounds, including bromazepam (Tateishi & Shimizu, 1976) and chlorotrifluoroethylene (Bhattacharya & Gandolfi, 1981) are cleanly acted upon by a mammalian β-lyase. Both liver and kidney are well endowed with cysteine conjugate β-lyase (Bhattacharya & Schultze, 1967; Dohn & Anders, 1982), but the enzymes in the two tissues differ in that the kidney protein does not react with antibody to the liver enzyme (Stevens & Jakoby, 1983).

Although the pathway does function, its biological significance and the extent to which xenobiotics utilize it, remain to be clarified.


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Glucocorticoid sulphotransferases in rats and other animal species

SANFORD S. SINGER
Department of Chemistry, University of Dayton, Dayton, OH 45469, U.S.A.

In recent years we have described sulphotransferases I, II and III (STI, STII and STIII), the rat liver enzymes (Singer et al., 1976, Singer, 1978a) that catalyse conversion of glucocorticoids into their 21-sulphates (for reviews see Singer, 1982, 1983). The probable importance of the enzymes is underscored by their relationships to aging (Singer & Bruns, 1978), hypertension (Singer et al., 1977), enzyme induction (Singer & Litwack, 1971), Litwack & Singer, 1972; Singer et al., 1975) and diabetes (Singer et al., 1981). These relationships complement reports suggesting that glucocorticoid sulphates are involved in control of corticosteroid metabolism (Gustafsson & Ingelman-Sundberg, 1974, Ingelman-Sundberg, 1976), hypertension (Kornel & Mitschak, 1965, Turcotte & Silah, 1970), diabetes (Stancakova et al., 1978) and cancer (Ghosh et al., 1973; Rose et al., 1975). This paper will (1) describe facile STI purification and comparison of STI with homogenous STIII, (2) summarize endocrine control of glucocorticoid sulphotransferase production, (3) indicate interrelationships between STI, STII, STIII and other relevant sulphotransferases, and (4) introduce our exploration of cortisol sulphation in non-rat species.

Facile STI purification and comparison of STI with homogenous STIII

We recently designed a facile method for STI purification to replace a cumbersome method (Singer, 1979) that yielded small amounts of 1000-fold purified STI. Initial development of the method was mentioned elsewhere (Lewis & Singer, 1976). It is a simple two-step procedure that recovers nearly half of the STI in rat liver as an approx. 2000-fold-purified fraction.

The first purification step was DEAE-Sephadex chromatography of cytosol prepared (Singer et al., 1976) from livers of female Sprague-Dawley rats (Charles River, Wilmington, MA, U.S.A.). Cytosol, 45-50 ml, was applied to 8.0 cm x 50 cm DEAE-Sephadex A-50 columns prewashed with 50 mM-Tris/250 mM-sucrose/3.0 mM-mercaptoethanol, pH 7.5 (Buffer I), to bring the effluent pH to 6.9-7.2. Columns were eluted with linear gradients composed of 2.8 litres each of Buffer I and Buffer I/400 mM-KCl. Elution rates and chromatographic profiles were as reported previously (Singer, 1979), with STI eluting prior to STII and STIII. Sulphate assays (see Singer et al., 1976) used 40 μM-cortisol and 240 μM-3'-phosphoehydinosine 5'-phosphosulphate. STI was 16.5-33.7% of the cortisol sulphotransferase activity recovered from columns (16 experiments). After correction of the specific activity of cytosol for STI content (multiplication by the fraction of recovered enzyme activity present as STI), the ion-exchange step was found to yield 21.1-32.3-fold-enriched STI preparations compared with cytosol. Preparation of cytosol from liver homogenates (Singer, 1979) purified its cortisol sulphotransferase activity fivefold. Therefore STI recovered by DEAE-Sephadex chromatography was enriched 133 ± 26 fold compared with homogenates. The procedure recovered 88.1-98.9% of the STI in homogenates. Use of frozen liver, or cytosol stored on ice for more than 1-2 h, reduced the yield of STI greatly.

The second preparative step was adenosine 5’-di-phosphate (PAP)-agarose chromatography. Here 2.0 cm x 2.3 cm PAP-agarose columns (PL Biochemicals, Milwaukee, W1, U.S.A.) were washed with 600 ml of Buffer I and pooled STI-containing fractions from DEAE-Sephadex chromatograms (400-550 ml) were applied over 2-3 h. Then columns were eluted overnight with linear gradients composed of 120 ml each of Buffer I and Buffer 1/12 mM-ADP. STI appeared as a sharp peak near the midpoint of each chromatographic profile. The procedure gave 12.5-23.50-fold STI purification, and 45-56% of the enzyme activity applied to the columns was recovered. Total STI purification was 2210 ± 400-fold compared with homogenates. PAP-agarose could be re-used three to five times.

Disc electrophoresis of the earlier STI preparation (Singer, 1979) showed that it contained nearly equal amounts of two protein bands. The 2210 ± 400-fold-purified STI was almost free of the ‘slower’ band. The enzyme, M, 160000, was shown to be composed of 28000-M, subunits by the method of Laemmli (1970). This indicated that STI was probably a hexameric protein. Its Km values for cortisol and 3'-phosphoehydinosine 5'-phosphosulphate were 7 and 11 μM respectively, at pH 6.8 [the routine assay pH for reasons previously described (Singer et al., 1976)]. The mechanism of cortisol sulphation at that pH was Theorell-Chance by criteria outlined by Plowman (1976). The pH optimum and K,, values for cortisol and 3'-phosphoehydinosine 5'-phosphosulphate were 7 and 11 μM respectively, at pH 6.8 [the routine assay pH for reasons previously described (Singer et al., 1976)].