Sulfation and Glucuronidation as Competing Pathways in the Metabolism of Hydroxamic Acids: The Role of N,O-Sulfonation in Chemical Carcinogenesis of Aromatic Amines

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Aromatic amines can be metabolized by N-acetylation and N-hydroxylation to hydroxamic acids; these subsequently are conjugated to form the N,O-sulfonate and N,O-glucuronide conjugates. The N,O-sulfonates are highly labile metabolites that generate reactive intermediates involved in the covalent binding of the parent compound to protein, RNA and DNA, as well as to low molecular compounds like glutathione. This paper discusses methods used to decrease sulfation in vivo, and thereby to enhance the formation of the more stable N,O-glucuronides from N-hydroxy-2-acetylaminofluorene and N-hydroxy-4-acetylamino-4'-fluorobiphenyl. Acetaminophen pretreatment decreases the sulfate availability, but results in many side effects that compromise the analysis of the results. An 8% casein diet reduces the sulfate availability in the rat to approximately 20% of control and thus offers an effective approach to decrease sulfation.

The most effective selective inhibition of sulfation is by pentachlorophenol, which very strongly reduces N,O-sulfonation of both hydroxamic acids, and selectively inhibits the formation of DNA adducts that have retained the N-acetyl group. This inhibitor and the related 2,6-dichloro-4-nitrophenol can be employed to study the role of sulfation of hydroxamic acids in initiation and promotion of tumor formation by aromatic amines.

Introduction

An important pathway in the conversion of aromatic amines to ultimate carcinogenic metabolites is N-acetylation followed by N-hydroxylation to form the hydroxamic acid derivatives; these, subsequently, are substrates for conjugation (1, 2). The main products are the relatively stable glucurononides and the more labile sulfate conjugates of the N-hydroxy group (2-4). Since the spontaneous breakdown of the N,O-glucurononides is much slower than that of the N,O-sulfonates, the chemical mechanisms of breakdown of these conjugates can more easily be studied with the N,O-glucurononides. Of course, it has to be kept in mind that the mechanisms may be different for the various conjugates. The sulfate conjugates are very prone to rapid, spontaneous rearrangements and, in general, are very labile in aqueous media, even at pH 7 (2, 5). However, the more stable glucuronide conjugate of N-hydroxy-2-acetylaminofluorene (N-hydroxy-2-AAF) can be isolated, and the properties of the reactive intermediate formed during its breakdown have been studied in vitro (2, 3). Similarly, the breakdown products of the glucuronide conjugate of N-hydroxyphenacetin could be characterized completely (5). Other relevant reactions that have been studied extensively, especially with N-hydroxy-2-AAF, are transacetylation, acetylation and deacetylation (6, 7). In addition, the N,O-glucuronide of N-hydroxy-2-AAF may be attacked by microsomal deacetylases which results in the formation of a reactive N,O-glucuronide of N-hydroxy-2-aminofluorene (8).

The sulfate conjugates of various hydroxamic acids have been implicated in carcinogenesis by those compounds (4). Early evidence came from work of the group of De Baun and the Millers (9, 10) and of King and Phillips (11). However, it has turned out to be rather difficult to prove unambiguously a role of sulfation in the carcinogenesis by N-hydroxy-2-AAF, because other very reactive metabolites of
this hydroxamic acid might be ultimate carcinogens (12). One of the main reasons is probably that no highly selective inhibitors of sulfation and/or glucuronidation were available to evaluate the role of these conjugations in carcinogenesis by aromatic amines or hydroxamic acids. In this paper we want to review the available methods to change, more or less specifically, the balance between glucuronidation and sulfation. This can be done either at the level of sulfate availability (by dietary methods or nonselective drugs) or at the level of the conjugating enzymes through selective inhibition of sulfation by pentachlorophenol (PCP) and 2,6-dichloro-4-nitrophenol (DCNP).

It seems relatively easy to study the involvement of glucuronides in the carcinogenicity of a compound, because usually the glucuronides can be isolated and subsequently tested in vivo. Thus, the role of the N-glucuronide of N-hydroxy-2-naphthylamine in bladder carcinogenesis (13) can be evaluated directly. However, if a glucuronide is only effective inside the cell, such studies may not be possible since most likely the glucuronides will be taken up only slightly by cells due to the poor lipid solubility of these compounds. Only uptake in hepatocytes may be efficient, as shown by the fact that glucuronides are in general rapidly excreted in bile in many species after intravenous administration (14). Hydrolysis of glucuronides usually does not occur in the body when they are injected intravenously (15).

**Dietary Methods to Decrease Sulfate Availability**

It would appear to be relatively easy to decrease sulfation by a reduction of sulfate availability by alteration of the diet. However, it turns out that this is more complicated than it looks at first sight. First of all, during fasting for several days, serum sulfate does not decrease, presumably because enough sulfate is provided by catabolism of cysteine, which is not required for protein synthesis, to inorganic sulfate (16). Only the amount of sulfate excreted in urine decreases under those conditions, which indicates that the rate at which sulfate becomes available in the body has been lowered considerably. Surprisingly, the steady-state rates of glucuronidation and sulfation of a phenolic substrate, harmol, were almost unaffected in rats fasted for 3 days (17).

Feeding a diet that is low in sulfur-containing amino acids similarly has little effect on serum sulfate (Table 1). The reason may be that at very low methionine (and no cysteine) in the food, growth stops and a catabolic state, comparable to fasting, follows; indeed, the urinary excretion of sulfate had been reduced by more than 50% (Mulder and Glazenburg, unpublished data).

The only diet that was highly effective in decreasing serum sulfate was an 8% (w/v) casein diet that, after 4 days, reduced the serum sulfate concentration in rats from the normal value of approx. 0.9 mM to 0.1-0.3 mM. This indicates a very effective decrease in sulfate availability. The first few days on this diet the rats lose some weight, but after 3 days they resume growth. When sulfate is infused intravenously in these animals, they still are capable of conjugation with sulfate and glucuronate at normal rates after 4 days on this diet. Therefore, the conjugating systems are completely unaffected under these conditions (apart from the cofactor availability).

Additional sulfate or the sulfate precursor L-cysteine added to the food had little effect on serum sulfate in rats (Table 1): the amount absorbed from the gut is eliminated continuously by the kidneys, so that no increased serum sulfate concentration results (16). Of course, if much of the endogenous sulfate is consumed by sulfation of a substrate, additional sulfate (or cysteine) in the food would replenish this loss of sulfate.

Sulfate required for N,O-sulfonation is not elimi-

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**Table 1. Effect of dietary changes on sulfate availability in the rat in vivo.**

| Diet                      | Serum sulfate, mM | Urinary sulfate μmole/24 hr/kg | Reference |
|---------------------------|-------------------|-------------------------------|-----------|
| Control                   | 0.85 - 1.0        | 1800 - 2000                   | (16)      |
| 8% Casein (for 4 days)    | 0.1 - 0.3         | 150 - 200                     | (16)      |
| Synthetic†                |                   |                               | b         |
| 100 Cys/100 Met           | 1.07              | 1900                          |          |
| 25 Cys/25 Met             | 1.20              | 800                           |          |
| 0 Cys/25 Met              | 1.20              | 650                           |          |
| 25 Cys/25 Met + 150 sulfate | 1.10            | 6000                          |          |
| Fasting (for 3 days)      | 0.94              | 1000                          | (16)      |

*The diet consisted of pure amino acids, peanut oil, corn starch, vitamins and minerals; the composition is almost identical to that reported elsewhere. (18). 100 Met means 22 mmole L-methionine per kg of the diet; 100 Cys means 26 mmole L-cysteine/kg food; 150 sulfate means 36 mmole sodium sulfate/kg food. These diets were given 8 consecutive days.

†Data of Glazenburg et al., in preparation.
nated from the body because the conjugates break down rapidly, inside the cell, and return the sulfate group back to the cellular sulfate pool. Only when a high dose of cysteine or sulfate is given orally as a single dose does serum sulfate increase for a prolonged period of time (16, 19, 20).

In conclusion, sulfate availability can be decreased by feeding rats an 8% casein diet, and it can only be increased when high doses of sulfate or cysteine are given acutely.

**Acetaminophen Pretreatment to Decrease Sulfate Availability In Vivo**

Many substrates of sulfation can deplete sulfate in the body when they are given in high doses, for instance, salicylamide, acetaminophen (paracetamol) and harmol (21-23). Several investigators in the field of aromatic amine carcinogenesis have employed acetaminophen to elucidate the role of sulfation in the generation of ultimate carcinogenic metabolites (24-27). The idea was that acetaminophen would deplete sulfate selectively and thereby decrease sulfation of, for instance, N-hydroxy-2-AAF. Adding inorganic sulfate or cysteine to the food would restore sulfate availability to the normal level. However, while high doses of acetaminophen do cause a decrease in serum sulfate, there is also much unconjugated acetaminophen present at the same time (16, 28). It has been shown by various groups that acetaminophen may have many other effects besides decreasing sulfate (27), such as an inhibition of transacetylation of N-hydroxy-2-AAF (28). Therefore, this is certainly not a method of choice, and results from these types of experiments should be analyzed with much caution.

Moreover, the addition of sodium sulfate or cysteine to the diet did not result in a rise in the plasma sulfate concentration in the rat (19), although it may restore the sulfate levels to normal in acetaminophen-pretreated rats.

**Selective Inhibition of Sulfation by Pentachlorophenol (PCP) and 2,6-Dichloro-4-Nitrophenol (DCNP)**

In 1977 two selective inhibitors of sulfation were discovered that had no effect on glucuronidation: pentachlorophenol (PCP) and 2,6-dichloro-4-nitrophenol (DCNP) (29). PCP strongly inhibited the in vitro sulification of N-hydroxyphenacetin and N-hydroxy-2-AAF, while DCNP was less effective (29, 30). The mechanism is reversible competitive inhibition with respect to the acceptor substrate (31, 32). The inhibition of sulfation by these compounds has recently been reviewed (33).

Pure PCP has relatively low toxicity (34), apart from its strong uncoupling action on oxidative phosphorylation. However, in the rat in vivo the inhibition of sulfation seems to occur at PCP concentrations that do not yet affect oxidative phosphorylation. Since PCP has a rather long half-life (6-24 hr) (34, 35), it seems a promising drug for use in studies on the role of sulfation in carcinogenesis by aromatic amines. It can be administered with the food or dissolved (as sodium salt) in the drinking water. If it is given for a prolonged period of time at 1.4 mM in the drinking water, the plasma levels of PCP are approximately 100 µm. Alternatively, it can be given as a single intraperitoneal injection or as sodium salt in osmotic micropumps that can be implanted subcutaneously. Very high infusion rates can be given by a single osmotic minipump during two weeks: 90 µmole/hr, also resulting in a plasma concentration of 100 µM (35). Therefore, it is relatively easy to ensure a high plasma level of PCP for a prolonged period of time as required in experiments in which sulfation has to be inhibited for several weeks.

PCP pretreatment resulted in pronounced changes in the metabolism of N-hydroxy-2-AAF and the related hydroxamic acid N-hydroxy-4'-fluoro-4-acetylaminobiphenyl (N-hydroxy-FAAB) (30, 36). As shown in Table 2, the formation of the N,O-glucuronide conjugates was greatly increased. Although the eventually formed N,O-sulfones could not be measured directly because of their extreme lability, the above findings seemed to suggest that sulfation of the hydroxamic acids at the N-hydroxy group is inhibited, and that a compensatory increase in glucuronidation has occurred (30, 36). Indeed, when the metabolism of N-hydroxy-2-AAF was determined in the isolated perfused rat liver, the omission of sulfate from the perfusion medium resulted in a similar, pronounced increase of the N,O-glucuronide production, which could not further be increased by the

| Substrate                  | Control | PCP  | DCNP |
|----------------------------|---------|------|------|
| N-Hydroxy-2-AAF            | 26.5 ± 1.6 | 47.4 ± 1.9 | N.D. |
| N-Hydroxy-FAAB            | 18.0 ± 0.4 | 39.4 ± 0.8 | 23.8 ± 1.7 |

*The dose of both substrates was 60 µmole/kg (IV); bile and urine were collected for 4 hr after injection (30, 36). The rats were pretreated 45 min before the injection of the substrate with PCP or DCNP (or the solvent in controls) at 40 µmole/kg (I.P.). The mean for four rats ± SEM are given; N.D. means not determined.
addition of PCP to the perfusion medium (30). If, therefore, the increase in N,O-glucuronide conjugate excreted above the control level in the presence of PCP was taken to be due to the inhibition of N,O-sulfonation, approximately 20-40% of the dose of N-hydroxy-2-AAF employed in the study of Meerman et al. (30) was converted to the reactive N,O-sulfonate conjugate. For N-hydroxy-FAAB this was ca. 20% (36).

Another indication of the generation of reactive intermediates can be gained from the amount of the substrate that becomes covalently bound to protein, RNA and DNA. It should be realized that sulfation is not the only pathway to form a reactive intermediate from hydroxamic acids, since deacetylation and transacetylation may also yield reactive species. Pentachlorophenol inhibited the covalent binding of N-hydroxy-2-AAF and N-hydroxy-FAAB to the total macromolecular fraction of the liver (30, 36). For N-hydroxy-FAAB the same was found for the kidney, also a target organ for carcinogenesis by this compound but not in the spleen (not a target organ) PCP had no effect on covalent binding (Table 3).

Table 3. Effect of PCP and DCNP on covalent binding of [9-14C]-N-hydroxy-2-AAF and [ring-3H]-N-hydroxy-FAAB to macromolecules in the liver, kidney and spleen of the rat.*

| Adduct to guanine | Control | PCP | DCNP |
|-------------------|---------|-----|------|
| N-Hydroxy-2-AAF | Liver DNA | 162 ± 20 | 132 ± 12 | 103 ± 4 |
|                   | Liver RNA | 301 ± 27 | 120 ± 7 | 155 ± 12 |
|                   | Liver protein | 472 ± 42 | 168 ± 32 | 224 ± 55 |
| N-Hydroxy-FAAB | Liver | 530 ± 31 | 290 ± 25 | 505 ± 32 |
|                   | Kidney | 897 ± 28 | 561 ± 67 | 931 ± 84 |
|                   | Spleen | 179 ± 48 | 202 ± 21 | N.D. |

*The dose and pretreatments were as in Table 2. Means ± S.E.M. are given; N.D. means not determined. For covalent binding to DNA and RNA, the binding as pmole/mg DNA and RNA is given; for covalent binding to protein and total covalent binding to all macromolecules (for N-hydroxy-FAAB) it is expressed per milligram protein (30, 36).

Further work with N-hydroxy-2-AAF showed that PCP prevented primarily covalent binding of [9-14C]-labeled N-hydroxy-2-AAF to protein, which accounts for most of the covalent binding in the liver. However, binding to DNA was also decreased (Table 3). Later (37) it was shown that the DNA adducts which retained their N-acetyl group were decreased selectively by PCP, while the deacetylated adducts were unaffected (Table 4). Since sulfation leads only to adducts that retain their N-acetyl group this PCP effect seems to indicate that the greater part of these adducts are formed by sulfation (37).

Table 4. Effect of PCP pretreatment on the formation of various DNA adducts to guanine from N-hydroxy-2-AAF in rat liver DNA.*

| Adduct to guanine | Controls | PCP-treated |
|-------------------|----------|-------------|
| N-Acetylated adducts | | |
| N2 - AAF | 2.7 ± 0.4 | 1.4 ± 0.1 |
| C8 - AAF | 23.7 ± 4.1 | 6.6 ± 0.7 |
| De-acetylated adducts | | |
| C8 - AF | 40.1 ± 6.2 | 53.0 ± 3.8 |

*Rats were pretreated with PCP (40 μmole/kg, IP) or with solvent; 45 min later they received an injection (IV) of 60 μmole/kg [ring-3H] N-hydroxy-2-AAF. Means ± S.E.M. are given for four or five rats. N2 and C8 indicate adducts through the N2 and C8 of guanosine, respectively (37).

Another property of N-hydroxy-2-AAF is its hepatotoxicity. Specifically the periportal "zone 1" cells are damaged (38, 39), and lymphocyte infiltration in this area is observed after 1 day. Both PCP and DCNP prevented this effect of N-hydroxy-2-AAF very effectively (39), indicating that this effect is related to the sulfation of N-hydroxy-2-AAF. The release of transaminases from the liver after N-hydroxy-2-AAF and its prevention by PCP and DCNP confirmed the protective effect of the latter compounds (39). It remains to be seen whether or not the cytotoxic effect is somehow involved in promotion of the carcinogenic action of 2-AAF.

**Study of the Role of Sulfation in Isolated Systems**

Although it is difficult to evaluate the role of sulfation in the generation of unstable, reactive intermediates in vivo, it is easier in isolated systems such as the perfused liver or isolated hepatocytes. These can be perfused or incubated in the absence of inorganic sulfate so that the only source of sulfate is that which is available in the liver. It should be realized that the liver can generate its own sulfate from sulfoxidation of cysteine or from breakdown of sulfated macromolecules such as glycosaminoglycans. Therefore, sulfation will certainly continue at a low rate even when inorganic sulfate and the sulfur-containing amino acids are completely deleted from the system.

Sometimes even the results with such systems are still hard to explain. For example, omission of sulfate from the incubation medium decreased covalent binding of N-hydroxy-2-AAF to protein (40), but had no effect whatsoever on covalent binding of 2-AAF (41).
Role of Glutathione in Detoxication of the Reactive Intermediates Generated from Acetylamino fluorene

When the sulfate conjugate of N-hydroxy-2-AAF breaks down, it generates an electrophilic AAF derivative that can bind to only nucleophilic groups in protein, RNA or DNA, but also to the -SH group of glutathione. The chemical reaction between N-acetoxy-2-AAF and glutathione in vitro yields four glutathione conjugates, at the 1-, 3-, 4- and 7-positions of the fluorene ring (42). All of these retained the N-acetyl group. Glutathione conjugates without this N-acetyl group are formed if, for instance, nitrosofluorene is reacted in vitro with glutathione (43). In the rat, in vivo, the 1- and 3-glutathionyl-AAF conjugates are formed from N-hydroxy-2-AAF. There was no evidence in preliminary experiments for the formation in vivo of deacetylated glutathione conjugates (Meeran, Kadlubar and Mulder, unpublished results). PCP pretreatment decreased the formation of both glutathione conjugates from N-hydroxy-2-AAF in vivo considerably, indicating that a major part of these conjugates had been formed through sulfation.

Similar glutathione conjugates resulted from the spontaneous breakdown of the N,O-glucuronide conjugate of N-hydroxy phenacetin (5).

As yet it is unclear why glutathione, while still available, cannot detoxify all of the reactive intermediate formed from N-hydroxy-2-AAF in vivo, so that much becomes covalently bound to protein and DNA whereas glutathione protects completely against paracetamol toxicity. Some reasons for this have been discussed elsewhere (44).

Sulfation of Hydroxamic Acids: Initiation or Promotion?

It has been suggested that sulfation of N-hydroxy-2-AAF is not responsible for initiation, but for tumor promotion, while initiation may be due to a different metabolite of N-hydroxy-2-AAF (45, 46). This promotion might be related to the hepatotoxic action that is the consequence of sulfation of N-hydroxy-2-AAF (44), which leads to a regenerative growth of the liver. To study this, the inhibitory action of PCP on sulfation can very conveniently be used in various test systems to further analyze the role of sulfation in initiation and promotion by AAF. Results of these types of experiments will become available in due time.

The research from our own laboratory has been supported by a grant from the Dutch Cancer Foundation (Koningin Wilhelmina Fonds).

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