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# Availability of plasma sulfate for conjugation of salicylamide in dogs*

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A failure of sulfoconjugation of phenolic substrates to increase in proportion to dose, observed in several species [1], could be due to a limitation in the rate of any one, or more, of several steps of sulfoconjugation [2] or to depletion of body stores of cosubstrate precursors [3–5]. In contrast to humans, sulfoconjugation of salicylamide in dogs shows this kinetic behavior even when plasma sulfate concentrations are normal [6] or increased [2]. Thus, one might hypothesize that transport into or activation of sulfate at the sites of sulfoconjugation could be responsible for limiting the rate of metabolism after increasing doses of the drug in the dog.

We report here studies designed to determine the source of sulfate used for the sulfoconjugation reaction and the rate of incorporation of plasma inorganic sulfate into salicylamide given intravenously to dogs. The plasma inorganic sulfate pool was labeled with [$^{35}$S]sulfate. Then the incorporation of radiolabeled sulfate into salicylamide-sulfate was determined by comparing the specific activities of inorganic sulfate in plasma and salicylamide-sulfate in urine.

## *Materials and methods*

*Chemicals.* [$^{14}$C]Salicylamide [2] was synthesized by ICN (Irvine, CA). [$^{35}$S]Sulfate (1070 Ci/mmole, primarily as the ammonium salt) was purchased from Amersham (Arlington Heights, IL).

*Animals.* Three conditioned male mongrel dogs weighing 20–26 kg were studied using the experimental techniques described previously [2].

*Rate of appearance of salicylamide-sulfate in the plasma.* [$^{14}$C]Salicylamide, 7.3 μmoles/kg (sp. act. = 275 μCi/mmole), in 10 ml normal saline, was administered intravenously to a dog over 1 min. Blood samples were collected at 1, 2, 3, 4, 5, 6, 8, 10, 13, 16, and 22 min. Plasma concentrations of [$^{14}$C]salicylamide-sulfate were measured.

*Fate of administered salicylamide-[$^{35}$S]sulfate.* To prepare salicylamide-[$^{35}$S]sulfate, 40 μCi of inorganic [$^{35}$S]sulfate and 32 μmoles/kg of salicylamide were administered in the same solution (45 ml 0.9% NaCl) intravenously to a dog over 1 min. Salicylamide-[$^{35}$S]sulfate was extracted from the 24-hr urine collection and counterextracted as described previously [7].

The isolated salicylamide-[$^{35}$S]sulfate (containing 40 μmoles of salicylamide-sulfate) was given intravenously over 17.5 min to a different dog. Urine was collected for 6 hr. The specific activity of salicylamide-[$^{35}$S]sulfate, extracted from the urine, was compared with that of the administered compound.

*Rate of incorporation of [$^{35}$S]sulfate into salicylamide-sulfate.* Normal saline containing unlabeled salicylamide, 7.3 μmoles/kg, and inorganic [$^{35}$S]sulfate, 10 μCi, was given intravenously (10 ml) to three dogs over 30 sec. Blood samples were obtained at 0, 1, 2, 3, 4, 5, 6, 9, 11, 14, 18, 22, 26, and 30 min and urine was collected over 6 hr. The specific activity of salicylamide-[$^{35}$S]sulfate in the urine was compared with that of inorganic sulfate in the plasma.

*Rate of incorporation of [$^{35}$S]sulfate into salicylamide-sulfate after a bolus of sodium sulfate.* To prelabel the metabolically-active sulfate pool, inorganic [$^{35}$S]sulfate, 10 μCi, was given intravenously over 30 sec to three dogs. Fifteen minutes later, an aqueous solution containing sodium sulfate, 1.13 mmoles/kg, and unlabeled salicylamide, 7.3 μmoles/kg, was infused simultaneously into two separate forelimb veins over 1.2 min. Blood and urine samples were collected and data were analyzed as described in the preceding section.

*Assays.* Concentrations of unlabeled salicylamide in the plasma were measured by high performance liquid chromatography (HPLC) [7]. Total [$^{14}$C] and [$^{14}$C]salicylamide concentrations in the plasma were measured as described previously [6]. The concentration of [$^{14}$C]salicylamide-sulfate was calculated by subtracting [$^{14}$C]salicylamide concentration from the total [$^{14}$C] concentration. Previous studies [6] indicated that at salicylamide doses of 36 μmoles/kg, or less, the sulfate conjugate constituted greater than 97% of the radioactivity appearing in the urine. Therefore, other [$^{14}$C]radiolabeled metabolites were unlikely to be present in the plasma.

Concentrations of inorganic [$^{35}$S]sulfate in the plasma were determined as follows. Plasma, 100 μl, was added to Aquasol (New England Nuclear, Boston, MA), 15 ml, for counting of total [$^{35}$S]-activity. Because molar concentrations of salicylamide-sulfate in the plasma after a 7.3 μmole/kg salicylamide dose (Fig. 1A) were less than 1% of molar concentrations of inorganic sulfate in the plasma (about 1 mM), the contribution of salicylamide-[$^{35}$S]sulfate to total plasma radioactivity was assumed to be less than 1% and, therefore, unimportant. [$^{14}$C]Salicylamide and [$^{35}$S]sulfate were not administered in the same studies.

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Plasma concentrations of inorganic sulfate were measured by the turbidimetric method described previously [2, 8]. Plasma for the standard curves was obtained from a dog in which plasma sulfate had been depleted 12 hr earlier by an oral dose of salicylamide, 0.58 mmole/kg. The sulfate concentration in this sample was assayed by ion chromatography.*

Concentrations of salicylamide-sulfate in the injection solution and in the urine were measured by ion-pair HPLC [7, 9]. One-minute fractions collected from the HPLC column were added to 10 ml of Aquasol to determine salicylamide-[$^{35}$S]sulfate concentrations. To show that no other [$^{35}$S]labeled endogenous substance appeared under the salicylamide-sulfate peak, 10 $\mu$Ci of inorganic [$^{35}$S]-sulfate was administered to a dog, and urine was subjected to the same analysis.

*Treatment of data.* Pharmacokinetic parameters were calculated as described previously [2]. Specific activity was determined by dividing the $\mu$Ci of [$^{35}$S]sulfate by the mmoles of salicylamide-sulfate in the sample.

## Results

*Rate of appearance of salicylamide-sulfate in the plasma.* The rates of disappearance of salicylamide and appearance of salicylamide-sulfate in the plasma demonstrate that sulfoconjugation of salicylamide was rapid and that the conjugate quickly left the cells in which it was formed (Fig. 1A). Concentrations of salicylamide-sulfate in the plasma reached a maximum value at 12 min and then declined with a half-life of about 75 min (Fig. 1B).

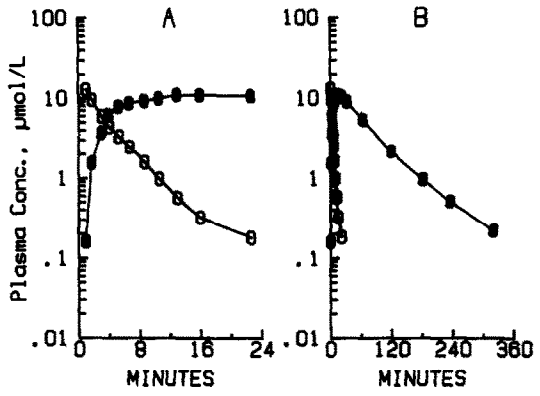


Fig. 1. Plasma concentrations of salicylamide (○) and salicylamide-sulfate (●) in a dog after an intravenous injection of [$^{14}$C]salicylamide (1.6 $\mu$Ci/kg, 7.3 $\mu$moles/kg). Molar concentrations were calculated from the concentration of drug or metabolite (dpm/ml) divided by the specific activity of the administered drug in dpm/$\mu$mole. Panel A: 0–24 min; Panel B: 0–360 min.

*Fate of administered salicylamide-[$^{35}$S]sulfate.* During the 6-hr collection period after salicylamide-[$^{35}$S]sulfate administration, 87% of the dose was excreted into the urine as salicylamide-[$^{35}$S]sulfate. If the administered salicylamide-sulfate had hydrolyzed and conjugated with unlabeled sulfate in the body (approximately 6.4 mmoles) [10], the specific activity of salicylamide-sulfate excreted in the urine would have decreased to 0.04 $\mu$Ci/mmole, i.e. less than 1% of that of the metabolite injected. However, the specific activity of the metabolite collected, 6.5 $\mu$Ci/mmole, was 102% of that of the salicylamide-[$^{35}$S]sulfate administered. Therefore, the metabolite did not undergo hydrolysis and reconjugation before its excretion in the urine.

*Rate of incorporation of [$^{35}$S]sulfate into salicylamide-sulfate.* Salicylamide, injected simultaneously with inorganic [$^{35}$S]sulfate, disappeared within minutes; half-life and clearance were 4.0 ± 2.2 min and 2.2 ± 0.4 l/min, respectively (mean ± S.D.). Plasma inorganic sulfate concentrations were 0.96 ± 0.16 mM before and 0.89 ± 0.11 mM 20 min after co-injection of salicylamide and inorganic [$^{35}$S]-sulfate. Over the time that salicylamide-sulfate was being formed, the specific activity of inorganic [$^{35}$S]sulfate in the plasma declined as the tracer distributed (Fig. 2). The 6-hr urinary recovery of the salicylamide dose as salicylamide-sulfate was 81 ± 2%. The specific activity of the salicylamide-sulfate in the urine, 2.36 ± 0.18 $\mu$Ci/mmole, was within the range observed for inorganic sulfate in the plasma by 2–3 min after salicylamide injection (Fig. 2).

*Rate of incorporation of inorganic [$^{35}$S]sulfate into salicylamide-sulfate after a bolus of sodium sulfate.* Under these experimental conditions, in three dogs, plasma salicylamide half-life and clearance were 5.4 ± 2.3 min and 4.5 ± 0.6 l/min, respectively. The rapid infusion of inorganic sulfate (1.13 mmoles/kg) increased plasma inorganic sulfate concentrations from 0.74 ± 0.16 mM before the infusion to 7.3 ± 0.9 mM 1 min after the infusion. Concentrations of sulfate declined, on the average, to 60% of this value over the next 20 min. The specific activity of inorganic [$^{35}$S]-sulfate in plasma dropped from 2.03 ± 0.07 $\mu$Ci/mmole just before salicylamide and sodium sulfate co-injection to 0.21 ± 0.03 $\mu$Ci/mmole 1 min after the injection. The specific activity increased gradually over the next 20 min to a value of 0.31 ± 0.05 $\mu$Ci/mmole.

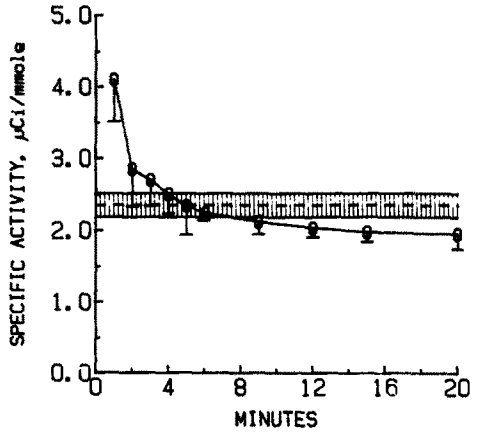


Fig. 2. Specific activity (mean ± S.D.) of plasma inorganic [$^{35}$S]sulfate versus time in dogs after intravenous co-injection of inorganic [$^{35}$S]sulfate, 10 $\mu$Ci, and salicylamide, 7.3 $\mu$moles/kg. The horizontal dashed line and shaded areas indicate the specific activity (mean ± S.D.) of salicylamide-sulfate excreted in the urine.

The 6-hr urinary recovery of the salicylamide dose as salicylamide-sulfate was 98 ± 5%. The specific activity of the salicylamide-sulfate recovered in the urine could not be accurately quantified because fractions collected from the HPLC column only contained near background levels of radioactivity. Even if the specific activity was at the limit of detection, 0.4 $\mu$Ci/mmole, the value would only be slightly greater than that of inorganic [$^{35}$S]sulfate in the plasma after dilution with unlabeled sodium sulfate (0.21 to 0.31 $\mu$Ci/mmole).

* Analyzed by Marilyn Morris in the Laboratory of Gerhard Levy, School of Pharmacy, State University of New York at Buffalo, Amherst, NY.

*Discussion*

Under the conditions of these studies, inorganic sulfate in plasma is in rapid equilibrium with the metabolically-active sulfate pool and is rapidly conjugated with salicylamide. These data in three dogs also demonstrate that the sulfate used for the reaction originates almost exclusively from inorganic sulfate in the plasma or from sulfate stores that are in rapid equilibrium with the plasma sulfate pool. These conclusions are deduced from the fact that the specific activity of salicylamide-sulfate excreted in the urine was not greatly different from that of inorganic sulfate in the plasma during the time that the metabolite was formed. These findings extend the observations made in other species. For example, after intravenous co-injection of inorganic [$^{35}$S]sulfate and harmol to rats, the specific activity of harmol-[$^{35}$S]sulfate appearing in the bile reaches a constant value within a few minutes [11]. In similar studies with phenol in mice, Herbai [12] demonstrated that the specific activity of phenol-[$^{35}$S]sulfate in the blood reaches a constant value at 15 min, the first time point measured.

Although the dose of salicylamide used in these studies was small, clearance of the 7.3 μmole/kg dose of salicylamide used here (2–5 l/min) was smaller than the clearances observed after tracer doses (36 nmoles/kg) of [$^{14}$C]salicylamide, 10–15 l/min [6]. The rapid equilibration of inorganic [$^{35}$S]sulfate in the plasma with the metabolically-active sulfate pool argues against these dose-dependent clearances being due to altered sulfate uptake or activation. However, an alternative interpretation of the data is that the entire active sulfate pool size is only a small fraction of the size of the salicylamide dose administered. Then, if active sulfate is consumed at a greater rate than it is formed, the unlabeled active sulfate pool could become depleted before a significant amount of the salicylamide dose is metabolized. Subsequently-formed metabolite would then, by necessity, reflect the specific activity of other available precursors, for instance, inorganic sulfate in plasma.

In the experiment in which the effective active sulfate pool was prelabeled with inorganic [$^{35}$S]sulfate, and salicylamide was given with a bolus of sodium sulfate, rapid equilibration between sulfate pools was again demonstrated. The specific activity of salicylamide-sulfate excreted in the urine reflected the rapid dilution of the specific activity of inorganic sulfate in plasma by the large bolus of sodium sulfate.

In summary, we measured the specific activities of both plasma inorganic sulfate and salicylamide-sulfate after [$^{35}$S]-sulfate was given intravenously to dogs. By comparing the specific activities, we demonstrated that, under the experimental conditions used, the plasma inorganic sulfate pool equilibrates rapidly with the effective active sulfate pool and provides the sulfate used for salicylamide sulfoconjugation.

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