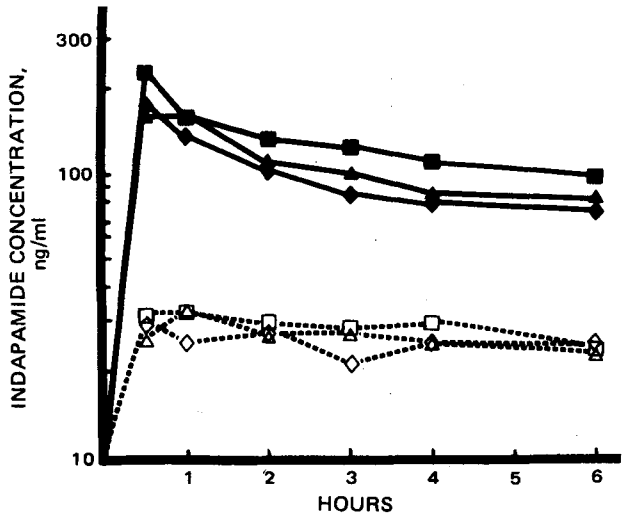

Figure 3—*Blood (■, ▲, ♦) and plasma (□, △, ◇) concentrations of indapamide following oral administration of 2.5 mg of indapamide to three subjects.*

be 2.0 ± 4.4 and 6.5 ± 5.7 ng/ml (mean ± *SD*) of apparent I, respectively. Based on these values, the sensitivity limit was considered to be ~20 ng of I/ml in both plasma and whole blood. A similar sensitivity limit was determined for urine samples.

Specificity—The specificity of the previous procedure for the measurement of unchanged I in urine was examined by high-pressure liquid chromatography (HPLC) and TLC (5). Blood concentrations of I from human subjects given the drug were measured both by the method described in this report and by an HPLC method that is under development. There was no significant difference between the measured I concentrations from the two procedures.

Applicability to Clinical Samples—The plasma and whole blood concentrations of I from three subjects who received an oral dose of 2.5 mg of I are shown in Fig. 3. The described procedure is adequate for monitoring the I concentrations in these media.

This method has several advantages over the previous method (5). The number of samples assayed per day is approximately doubled. The sensitivity is improved because of the better reproducibility of a continuous-flow system. In particular, a stable baseline is obtainable since the flowcell is not manipulated between samples as is a normal fluorescence cell during a manual procedure.

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Sulfoconjugation and Glucuronidation of Salicylamide in Isolated Rat Hepatocytes

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Abstract □ Sulfoconjugation and glucuronidation of salicylamide by isolated hepatocytes were examined with various concentrations of salicylamide and sodium sulfate. The ratio of sulfate to glucuronide formed changed markedly, depending on the concentrations of salicylamide and inorganic sulfate in the medium. The apparent K_m value of sulfoconjugation for salicylamide was 0.006 m*M*, while its V_{max} value varied depending on the concentration of inorganic sulfate (*e.g.*, 2.1 and 0.5 nmoles/min/10^6 cells at 1.2 and 0.5 m*M* inorganic sulfate, respectively). The K_m and V_{max} values of glucuronidation for salicylamide were 0.19 m*M* and 1.28 nmoles/min/10^6 cells, respectively, in the absence of sodium sulfate. Glucuronidation was suppressed in the presence of inorganic sulfate. The suppression could be attributable to the competitive consumption of salicylamide by sulfotransferase. Additional *in vivo* experiments revealed that an extra amount of salicylamide markedly lowered the blood inorganic sulfate levels of rats. The significance of the finding is discussed in conjunction with the variation of the V_{max} value of sulfoconjugation with the inorganic sulfate concentration.

Keyphrases □ Salicylamide—sulfoconjugation and glucuronidation □ Hepatocytes, rat—sulfoconjugation and glucuronidation of salicylamide □ Sulfate, inorganic—effect on conjugation and glucuronidation of salicylamide □ Conjugation—salicylamide, effect of inorganic sulfate

Numerous phenolic compounds are competitively metabolized *in vivo* to their conjugates by glucuronidation with uridine diphosphate (UDP) glucuronic acid and sulfoconjugation with 3′-phosphoadenosine-5′-phosphosulfate. In many cases, the sulfate is formed mainly at a low dosage while the glucuronide predominates at a high dosage (1–3).

This dose-dependent reversal of the sulfate/glucuronide ratio was explained by Levy and Matsuzawa (1) as follows. Blood inorganic sulfate can be consumed temporarily if a large amount of phenolic drug is administered and the 3′-phosphoadenosine-5′-phosphosulfate available for sulfoconjugation is limited, leading to a lower proportion of sulfate in the total conjugates. In fact, capacity-limited suppression of the sulfoconjugation can be overcome by simultaneous administration of drugs and sodium sulfate or cysteine to the subjects (4, 5). However, Weitering *et al.* (6) reported that the injection of phenol to rats did not lower the inorganic sulfate levels enough to elucidate the phenomenon. They emphasized that the reversed conjugation ratio found in *in vivo* experiments could be attributable to the different K_m values of sulfotransferase and UDPglucuronyltransferase.

The problems underlying these controversial proposals may be settled by a simpler *in vitro* experiment using an isolated hepatocyte system. Isolated hepatocytes maintain

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biochemically and structurally organized functions and allow easier kinetic study than *in vivo* systems (2). Their ability to generate endogenous cosubstrates as well as to catalyze sequential reactions is particularly useful for drug conjugation studies (7). In view of its established metabolic routes and clinical importance (1, 8), salicylamide was chosen as a model, and its conjugation in isolated hepatocytes was studied. Attention was focused on the effect of inorganic sulfate on conjugation at high and low salicylamide concentrations.

EXPERIMENTAL

Materials—The following materials were obtained commercially: collagenase[1] (*Clostridium histolyticum*), bovine serum albumin[2] (demineralized), amino acid mixture[3] (without L-glutamate), β-glucuronidase–arylsulfatase[4] (*Helix pomatia*, 9.3 IU of β-glucuronidase/ml, 5.9 IU of arylsulfatase/ml), β-glucuronidase[5] (calf, 13,000 UF/ml), salicylamide[6], and 2,6-dichloro-4-nitrophenol[7]. All other chemicals were reagent grade.

Preparation of Isolated Hepatocytes—Suspensions of isolated hepatocytes were prepared from male Wistar rats, 250–300 g, fed *ad libitum*, as described by Moldéus *et al.* (9). The oxygenator and buffer reservoir were jacketed for thermostatic control at 37°, and 13 m*M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid[8] and penicillin[9] (400 IU/ml) were added to the buffers (10). The yield of each preparation was 5–6 × 10^8 cells/liver, and the viability of fresh cells was 98–99% according to the lactic dehydrogenase latency test (9). The values were unchanged for 5 hr if the cells were suspended in albumin containing Krebs–Henseleit buffer at 4° saturated with 95% O_2–5% CO_2.

Incubation of Isolated Hepatocytes—Prior to incubation, the cells were washed twice with pH 7.4 Krebs–Henseleit buffer in which magnesium sulfate was replaced by magnesium chloride. To eliminate possible adsorption and binding of salicylamide conjugates (11), bovine serum albumin was not included in the buffer, although this omission caused deterioration of the cell viability to 95–96%. The cell suspension (7–15 ml/flask) was incubated in a rotating round-bottom flask at 37° under a stream of oxygen–carbon dioxide with 2 × 10^6 cells/ml unless specified otherwise. For the kinetic studies, different amounts of salicylamide and sodium sulfate were added to Krebs–Henseleit buffer, in which magnesium sulfate was replaced by magnesium chloride and supplemented with 10 m*M* glucose, amino acid mixture, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, and penicillin.

The viability of the isolated hepatocytes was checked periodically with the lactic dehydrogenase latency test (90–92% after 40 min of incubation). At intervals, samples were transferred to test tubes and boiled for 1 min to terminate the reaction. After centrifugation for 20 min at 2000 rpm, 1 ml of supernate was used for the conjugate determination.

Determination of Salicylamide Conjugates—Salicylamide sulfate and glucuronide were analyzed by the modified method of Veresh *et al.* (12) and Houston and Levy (13). One milliliter of sample was combined with 1.5 ml of 0.1 *M* acetate buffer (pH 5.0) and 6 ml of ethylene dichloride in a 12-ml glass-stoppered test tube, which was shaken vigorously for 15 min and centrifuged for 15 min at 2000 rpm. After removal of the organic layer by suction, 5 ml of ethylene dichloride was added to the tube and the extraction was repeated.

Subsequently, two parts of a 1-ml portion from the upper layer were taken into two tubes, one for the determination of glucuronide (Tube A) and the other for the determination of sulfate (Tube B). Salicylamide glucuronide was determined by adding 0.5 ml of β-glucuronidase and 1 ml of 0.1 *M* acetate buffer (pH 5.0) to Tube A and incubating for 40 hr at 37°. Salicylamide released by the enzymic hydrolysis was extracted with 6 ml of ethylene dichloride by shaking. After centrifugation, 4-ml portions of the organic layer were transferred to a test tube containing 5 ml of 0.1 *M* carbonate buffer (pH 11), which was shaken for 15 min and centrifuged for 10 min at 2000 rpm. The amount of salicylamide in the aqueous layer was determined fluorometrically using 325 and 420 nm for excitation and emission, respectively.

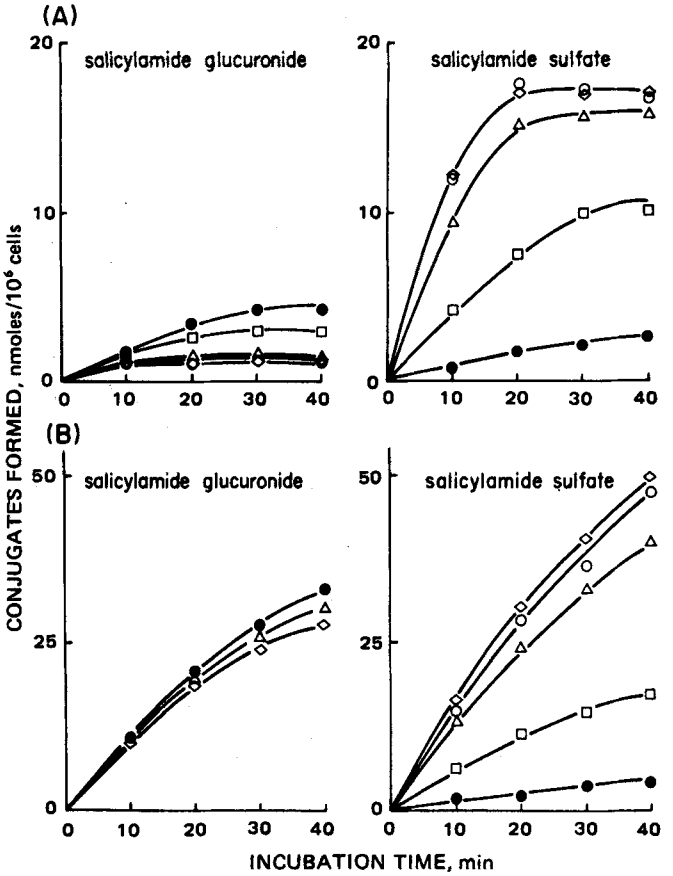

Figure 1—*Time courses of sulfoconjugation and glucuronidation of salicylamide in isolated rat hepatocytes at 0.05 m*M *(A) and 0.4 m*M *(B) salicylamide. Incubation was performed as described in the text in the absence (●) or presence of 0.1 (□), 0.5 (△), 1.2 (○), or 3.0 (◇) m*M *sodium sulfate.*

Next, the amount of salicylamide sulfate was determined by adding 0.1 ml of β-glucuronidase–arylsulfatase and 1 ml of 0.1 *M* acetate buffer (pH 5.0) to Tube B and incubating for 40 hr at 37°. By the same procedures used for Tube A, the total amount of salicylamide sulfate and glucuronide was determined, and the amount of the sulfate was calculated by subtracting the values obtained for Tube A from those for Tube B. Blank values were obtained in the same way, except that boiled liver cells were used. Incubation of isolated hepatocytes with different amounts of salicylamide and sodium sulfate was repeated two to three times, and the reproducibility was confirmed.

Assay of Inorganic Sulfate in Rat Serum—Male Wistar rats, 320–370 g, were anesthetized with pentobarbital sodium[10] before polyethylene tubing[11] was introduced into their right external jugular veins. Immediately after withdrawal of 2 ml of blood, 50 mg of salicylamide[12]/kg was administered. Subsequently, at 15-min intervals, 2 ml of blood was collected *via* the cannula and then centrifuged for 30 min at 4000 rpm using a serum separation aid[13]. The amount of inorganic sulfate in the serum was analyzed by turbidometry as described by Weitering *et al.* (6). Prior to the drug administration studies, it was confirmed that inclusion of 1 m*M* salicylamide in the serum, taken from an untreated rat, would not interfere with the determination of inorganic sulfate.

RESULTS AND DISCUSSION

The time courses of sulfoconjugation and glucuronidation of salicylamide by isolated hepatocytes at 0.05 and 0.4 m*M* salicylamide and

[1] Boehringer Mannheim GmbH., West Germany.
[2] Povite Producten N.V., Amsterdam, The Netherlands.
[3] Gibco minimal essential medium amino acid mixture (without L-glutamate), Grand Island Biochemical Co., Grand Island, N.Y.
[4] Calbiochem, San Diego, Calif.
[5] Tokyo Zoki Chemical Co., Tokyo, Japan.
[6] Nakarai Chemicals, Kyoto, Japan.
[7] Tokyo Kasei Co., Tokyo, Japan.
[8] Hepes, Nakarai Chemicals, Kyoto, Japan.
[9] Penicillin G potassium, Meiji Seika Co., Tokyo, Japan.
[10] Somnopentyl, Pitman-Moore, Washington Crossing, N.J.
[11] Intramedic PE60, Clay Adams, Parsippany, N.J.
[12] Salicylamide was dissolved in 1 *N* NaOH, and the pH was adjusted to 9.0 with hydrochloric acid. The final concentration of salicylamide was 20 mg/ml.
[13] Separaid, HATCO International, Los Angeles, Calif.

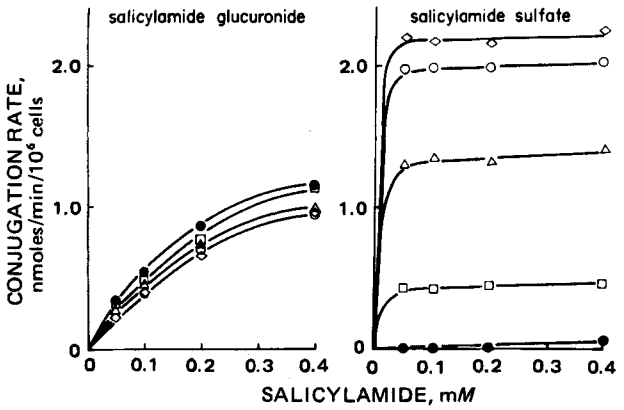

Figure 2—*Effect of salicylamide concentrations on the rate of salicylamide conjugation in isolated hepatocytes. The cells (1 × 10⁶ cells/ml) were incubated for 7 min at 37° with 3.0 (◇), 1.2 (○), 0.5 (△), or 0.1 (□) mM sodium sulfate or without (●) sodium sulfate.*

various inorganic sulfate concentrations are shown in Fig. 1. Apparently, a larger amount of salicylamide sulfate formed as the sodium sulfate concentration increased, whereas glucuronidation was suppressed at higher inorganic sulfate concentrations. Although the levels of conjugation varied, similar patterns of time courses were observed with the incubation system containing 0.1 or 0.2 m*M* salicylamide (not shown). An appreciable amount of salicylamide sulfate also was produced in the system without sodium sulfate, presumably due to the use of amino acids (*e.g.*, cysteine and methionine) in the medium as the source of sulfate, although the presence of contaminative inorganic sulfate in the cell isolation medium could not be ruled out. The ratio of conjugates, sulfate to glucuronide, changed markedly depending on the salicylamide concentration. For example, with 1.2 m*M* sodium sulfate, the sulfate to glucuronide ratio in a 10-min period decreased from 15 to 1.2 if the salicylamide concentration was increased from 0.05 to 0.4 m*M*.

Figure 2 shows the kinetics of conjugation by a 7-min incubation of isolated liver cells in the presence of different amounts of salicylamide and inorganic sulfate. The sulfoconjugation rate reached a maximum at low salicylamide concentration, while the glucuronidation rate increased progressively with increasing salicylamide concentrations. The effects of sodium sulfate on the two conjugation reactions were contrasting. The sulfoconjugation was accelerated by increasing inorganic sulfate, indicating the simultaneous increase of 3′-phosphoadenosine-5′-phosphosulfate, the cosubstrate for the conjugation, within the liver cells. On the other hand, the glucuronidation was suppressed by inorganic sulfate.

The reciprocal plots of [salicylamide glucuronide] against the reciprocal

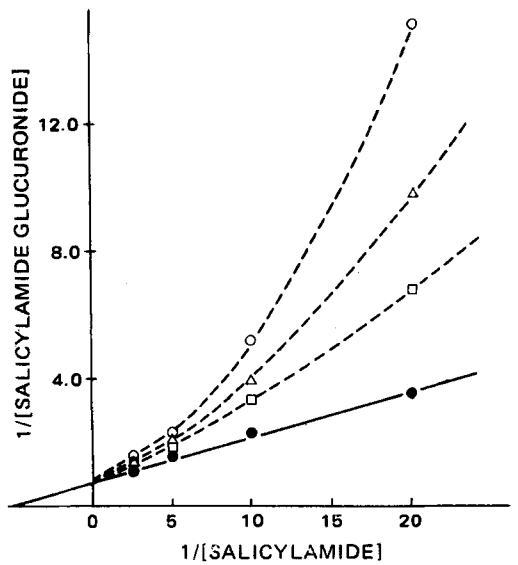

Figure 3—*Double reciprocal plots of salicylamide concentration (millimolar) against salicylamide glucuronide formed (nanomoles per minute per 10⁶ cells). The illustration was based on Fig. 2. The sodium sulfate concentration was 1.2 (○), 0.5 (△), 0.1 (□), or 0 (●) m*M.

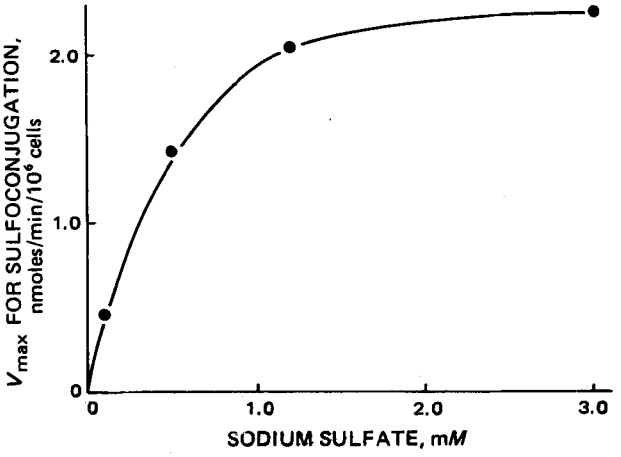

Figure 4—*Dependency of V_{max} values of salicylamide sulfoconjugation on inorganic sulfate concentrations.*

of [salicylamide] in the absence of sodium sulfate produced a straight line (Fig. 3), and the K_m and V_{max} values of glucuronidation for salicylamide were estimated to be 0.19 m*M* and 1.28 nmoles/min/10⁶ cells, respectively. Concave curves produced in the presence of sodium sulfate indicate the inhibitory effect of inorganic sulfate on glucuronidation. The effect was less pronounced at higher salicylamide concentrations and was not appreciable at V_{max}, as indicated by the intersection of the lines on the vertical axis (Fig. 3). Although inorganic sulfate appeared to interfere with glucuronidation in an unusual manner, inhibition by inorganic sulfate has not been reported to the knowledge of the authors.

Anomalous kinetics of salicylamide glucuronidation in the presence of sodium sulfate can be interpreted alternatively on the basis of localization and K_m differences of sulfotransferase and UDPglucuronyltransferase in isolated hepatocytes (14). The deviation from a straight line in Lineweaver–Burk (15) plots with increasing sodium sulfate concentration was probably due to the preferential use of salicylamide by sulfotransferase. When the concentration of salicylamide was low and that of inorganic sulfate was high, a large proportion of salicylamide, incorporated into the liver cells, could be used by sulfotransferase (which has a low K_m value for the drug) in cytosol. Therefore, the actual concentration of salicylamide available for glucuronyltransferase in the endoplasmic reticulum would have been much lower than the amount added to the medium. However, if the salicylamide concentration was high, the reaction rate of sulfotransferase would reach a maximum level, thus leaving some substrate for glucuronidation. Therefore, the ratio of sulfate to glucuronide conjugates would decrease as the substrate concentration increased.

Since an isolated hepatocyte system functions like an *in vivo* system in many respects (2, 16), this interpretation may be more likely. The apparent K_m and V_{max} values of sulfoconjugation for inorganic sulfate were 0.47 m*M* and 2.62 nmoles/min/10⁶ cells, respectively, as calculated

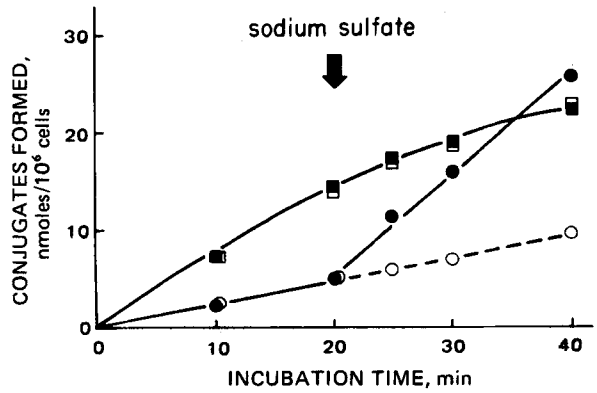

Figure 5—*Time courses of salicylamide conjugations: response to the increasing sulfate concentration from 0.1 to 1.2 m*M*. Isolated liver cells were incubated in the presence of 0.4 m*M* salicylamide and 0.1 m*M* sodium sulfate (○ and □). After 20 min of incubation, the concentration of sodium sulfate was increased to 1.2 m*M* (● and ■). Key: ○, ●, salicylamide sulfate; and □, ■, salicylamide glucuronide.*

Table I—Effect of a High Dose [a] of Salicylamide on Rat Serum Inorganic Sulfate

Minutes after Injection	n [b]	Sulfate Concentration [c], mM
0	7	0.72 ± 0.04
15	7	0.33 ± 0.08
30	7	0.16 ± 0.08
45	6	0.25 ± 0.12
60	4	0.43 ± 0.08

[a] Fifty milligrams of salicylamide/kg was intravenously administered. [b] Number of animals. [c] Mean sulfate concentration ± *SD*.

from typical Lineweaver–Burk plots. Since the K_m value of sulfoconjugation for salicylamide was very small, it was obtained with plots of $[S]/V$ *versus* $[S]$ (S, salicylamide; V, the rate of sulfoconjugation) (14) and was 0.006 mM when calculated by the least-squares method. The V_{max} values of sulfoconjugation for salicylamide varied, depending on the sodium sulfate concentration (Fig. 4). The effect of inorganic sulfate on V_{max} was remarkable at concentrations lower than 1.2 mM, indicating that the variation of inorganic sulfate in biological fluid would produce profound effects on sulfoconjugation of the drug *in vivo* since the level of inorganic sulfate in human serum is reported to vary from 0.2 to 0.7 mM (17).

Another feature of conjugation was a quick response of the sulfoconjugation reaction to exogenous inorganic sulfate (Fig. 5), suggesting rapid incorporation of inorganic sulfate into liver cells and biotransformation to its active form, 3′-phosphoadenosine-5′-phosphosulfate (18). This finding supports the clinical utility of sodium sulfate for reducing acute toxicity of certain drugs (5, 19, 20).

To study the postulated depletion of blood inorganic sulfate by ingestion of a large amount of phenolic drug (1), 50 mg of salicylamide/kg iv was injected to anesthetized rats, and the inorganic sulfate levels in blood serum were analyzed for 60 min (Table I). The inorganic sulfate concentration decreased to as low as 22% of the initial blood level 30 min after the administration and then gradually recovered. This recovery could have been attained by the formation of inorganic sulfate from amino acids and the release of inorganic sulfate from various tissues (21). The analytical procedure using barium chloride (6) seems to be pertinent in this study since similar results were reproduced by isotachophoretic assay of inorganic sulfate in the blood samples (not shown). Thus, the data in Table I and Fig. 4 show that the V_{max} values of salicylamide sulfoconjugation at 0.7 (initial blood level) and 0.16 mM inorganic sulfate (30 min after the injection) were 1.68 and 0.62 nmole/min/10^6 cells, respectively, which implies at most a 60% reduction of the sulfoconjugation reaction by an extreme salicylamide overdosage.

An additional experiment confirmed the effect of 2,6-dichloro-4-nitrophenol, an *in vivo* sulfoconjugation inhibitor (22), on sulfoconjugation of salicylamide in isolated hepatocytes. The conjugation was terminated instantaneously by 0.05 mM 2,6-dichloro-4-nitrophenol added to the system 10 min after incubation of the cells with 0.1 mM salicylamide and 1.2 mM sodium sulfate, whereas glucuronidation was slightly enhanced (not shown).

In conclusion, kinetic studies of the salicylamide metabolism using isolated hepatocytes allowed rational interpretation of previous conflicting *in vivo* observations on the metabolism of phenolic drugs, where dose-dependent fluctuation of the sulfate to glucuronide ratio was characteristic (1–3, 23). Available evidence suggests that at a low salicylamide dosage, a large proportion of the drug entering the cells is consumed preferentially by sulfotransferase in the cytosol and the effective salicylamide concentration for microsomal UDPglucuronyltransferase becomes very low. Consequently, the ratio of sulfate to glucuronide should be large. At high dosage, however, the ratio decreases because a larger amount of the drug is accessible to glucuronyltransferase, and glucuronidation occurs more actively. Furthermore, the observed variation of the V_{max} value of sulfoconjugation for salicylamide, depending on the inorganic sulfate concentration, and the reduction of blood inorganic sulfate levels by the administration of salicylamide will, in combination, give rise to the capacity-limited suppression of sulfoconjugation of the drug *in vivo*.

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