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Futile Cycling of a Sulfate Conjugate By Isolated Hepatocytes

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SUMMARY

The sulfate conjugate of the model compound 4-methylumbelliferone was taken up and hydrolyzed considerably more rapidly by isolated hepatocytes than was the glucuronide conjugate. Using intact hepatocytes or homogenates of hepatocytes, compounds were identified that either inhibited 4-methylumbelliferyl sulfate hydrolysis via arylsulfatase or impaired its uptake into cells. For example, sodium sulfate inhibited hydrolysis of 4-methylumbelliferyl sulfate by intact hepatocytes (half-maximal inhibition, 0.1 mM) but not by homogenates, suggesting a selective action on organic sulfate uptake at the plasma membrane. In contrast, cholesterol sulfate inhibited hydrolysis of 4-methylumbelliferyl sulfate by homogenates but not by hepatocytes, consistent with the hypothesis that cholesterol sulfate does not readily enter intact cells. Compounds that inhibited hydrolysis of 4-methylumbelliferyl sulfate by both isolated hepatocytes and microsomes include sodium sulfite (half-maximal inhibition, 0.1 mM), pregnenolone sulfate (half-maximal inhibition, 1 μM), and estrone sulfate (half-maximal inhibition, 10 μM). To test whether production of sulfate conjugates could be modified by agents affecting arylsulfatase in intact hepatocytes, we examined the effects of pregnenolone sulfate on the production of 4-methylumbelliferyl sulfate from 4-methylumbelliferone. Addition of pregnenolone sulfate (100 μM) to intact cells increased rates of 4-methylumbelliferone sulfate production and decreased the fraction of 4-methylumbelliferone converted into the glucuronide. Hydrolysis of 4-methylumbelliferyl sulfate by isolated microsomes was inhibited in a dose-dependent manner by adenosine 3′-phosphate 5′-phosphosulfate (PAPS) when cytosol, a source of sulfotransferase, was present. Furthermore, addition of low concentrations of PAPS (0.5 μM) to a reconstituted system of microsomes and cytosol impaired the formation of fluorescent product from 4-methylumbelliferyl sulfate until PAPS was consumed, indicating that futile cycling via arylsulfatase and sulfotransferase occurred. Subsequent futile cycling of free 4-methylumbelliferone and 4-methylumbelliferyl sulfate occurred upon repeated additions of PAPS and was prevented by sodium sulfite, an inhibitor of arylsulfatase. These results argue strongly that sulfate conjugate production within hepatocytes is regulated by futile cycling via sulfotransferase and arylsulfatase. Thus, drugs and endogenous substances that affect arylsulfatase may have marked effects on sulfate conjugate production by the liver.

Conjugation of drugs and xenobiotics by intact hepatocytes is a complex process influenced by a number of factors that include the availability of active intermediates such as PAPS and UDP-glucuronic acid (1, 2), the uptake of substrates, and the release of conjugated products (3), as well as the activities of specific transferases and hydrolases more commonly studied *in vitro* (4–6). Often overlooked in discussions of the regulation of drug metabolism is the possibility that futile cycling of conjugated substrates occurs via hydrolysis and resynthesis in the liver. Once conjugated by sulfate or glucuronic acid, xenobiotics and endogenous compounds can become substrates for arylsulfatase (EC 3.1.6.1) and β-glucuronidase (EC 3.2.1.31), which are present at relatively high activities in liver (6, 7).

Although studies of glucuronide and sulfate production by broken cell preparations (6, 7) suggested that futile cycling via specific transferases and hydrolases occurs, this possibility has not been evaluated critically in intact hepatocytes. Preliminary studies carried out in our laboratory suggested that futile cycling may be more important in the regulation of sulfate than of glucuronide conjugates (6). This was based on the observation that addition of sodium sulfite, an inhibitor of arylsulfatase (6), to homogenates of human liver incubated with PAPS increased rates of sulfation of hydroxycoumarin. On the other hand, saccharolactone, an inhibitor of β-glucuronidase, failed to increase glucuronide production (6). Factors regulating sulfate conjugate production need to be elucidated, because sulfation generally predominates at the low substrate concentrations that prevail under physiological conditions (8, 9). In addition to being a major route of xenobiotic metabolism, sulfation is also important in the metabolism of a number of hormones, including estrogen (10), catecholamines (11), and thyroxine

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ABBREVIATIONS: PAPS, adenosine 3′-phosphate 5′-phosphosulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

(12), which circulate primarily as sulfate conjugates *in vivo*. Sulfate conjugate production is also a critical step in the activation of a variety of carcinogens, e.g., acetyl aminofluorine (13).

The purpose of the present study was to evaluate the role of arylsulfatase in the regulation of sulfation of the model substrate 4-methylumbelliferone, in isolated hepatocytes and broken cell preparations from rat liver, to determine whether net sulfate conjugate production by the liver is influenced by futile cycling via arylsulfatase and sulfotransferase. 4-Methylumbelliferone was selected because it is highly fluorescent, allowing metabolism to be monitored easily. Results indicate that inhibition of arylsulfatase in intact cells as well as cell-free systems enhances the formation of 4-methylumbelliferyl sulfate from 4-methylumbelliferone. Thus, sulfate conjugate production in intact hepatocytes is regulated, at least in part, by hydrolysis and resynthesis (i.e., futile cycling).

Materials and Methods

Preparation of hepatocytes. Male Sprague-Dawley rats (150–300 g) used in these experiments were allowed free access to water and laboratory chow, and hepatocytes were isolated by a modification of the method described by Berry and Friend (14, 15). Briefly, rats were anesthetized with sodium pentobarbital (75 mg/kg, intraperitoneally) before perfusion of the liver for 5 min with 100 ml of calcium-free Krebs-Henseleit bicarbonate buffer containing 0.5 mM EGTA, 250 units of heparin, and 0.02% bovine serum albumin. Buffers used during the isolation were maintained at 37° and equilibrated with 95% O_2/5% CO_2, using a water-jacketed oxygenator (16). Perfusion using a recirculating system was continued *in situ* for 15 min, with 100 ml of buffer containing 30 mg of collagenase (Cooper Biochemicals). The liver was then removed and minced in 50 ml of buffer containing 0.02% bovine serum albumin. Hepatocytes were collected by filtration through two successive nylon mesh screens (Nitex 3-70-T and 3-305-T) and were washed twice by centrifugation (500 × *g* for 3 min) and resuspension. Viable hepatocytes were isolated by centrifugation through a 30% Percoll gradient (17). Hepatocytes were resuspended at 1×10^7 cells/ml in Krebs-Henseleit buffer containing 2% bovine serum albumin and were 95–98% viable, as indexed by trypan blue exclusion.

Preparation of microsomes and cytosol. Hepatocytes were homogenized in 10 volumes of 2 mM Tris·HCl, pH 8.1, containing 230 mM mannitol and 70 mM sucrose. The homogenate was centrifuged for 5 min at 500 × *g* to remove cell debris and nuclei, and the supernatant was centrifuged for 15 min at 15,000 × *g* to remove lysosomes and mitochondria. The resulting supernatant (1 ml) was then centrifuged for 5 min at 100,000 × *g* in a Beckman Airfuge, using an A-110 rotor. The supernatant fraction (cytosol) was removed, and the microsomal pellet was washed once in isotonic buffer and resuspended in Tris/mannitol/sucrose, at a protein concentration of approximately 20 mg/ml. The yield of microsomal protein was approximately 20 mg/g of wet tissue. Protein was determined according to the method of Lowry *et al.* (18).

Uptake and hydrolysis of 4-methylumbelliferyl sulfate by hepatocytes. 4-Methylumbelliferyl sulfate was stored as a 25 mM stock solution in 50 mM sodium acetate, pH 5.0, at −20°. Hepatocytes (1×10^6 cells/ml) were incubated with 4-methylumbelliferyl sulfate, at concentrations indicated in table and figure legends, at 37° in 4 ml of sulfate-free Krebs-Henseleit buffer containing 0.2% bovine serum albumin, 50 μM EDTA, 1 mM sodium ascorbate, and 12.5 mM HEPES, pH 7.4, in 25-ml plastic Erlenmeyer flasks. Incubations were performed at 37° in a shaking water bath under a 95% O_2/5% CO_2 atmosphere. Samples (0.5 ml) were removed at various times, as indicated in table and figure legends, and cells were pelleted by centrifugation at 15,000 × *g* for 1 min. Free 4-methylumbelliferone in the supernatant was measured fluorometrically (366 to 450 nm) in 1 ml of 0.02 M sodium carbonate buffer, pH 10.0. Because of the high fluorescence yield of 4-methylumbelliferyl at alkaline pH, as little as 10 pmol of substrate could be detected easily. 4-Methylumbelliferyl glucuronide was measured fluorometrically by incubation of samples with bovine liver β-glucuronidase (12.5 units) for 1 hr at 37°, in 50 μl of 0.2 M sodium acetate, pH 4.0, before addition of sodium carbonate. 4-Methylumbelliferyl sulfate was determined fluorometrically by incubation of an aliquot of the supernatant with 0.1 mg/ml β-glucuronidase/arylsulfatase mixture from *Patella vulgata* (Sigma) for 1 hr at 37°, in 50 μl of 50 mM sodium acetate, pH 5.0, containing 2 mM saccharolactone to inhibit β-glucuronidase. Stock solutions of saccharolactone were prepared just before use.

Glucuronidation and sulfation of 4-methylumbelliferone by intact hepatocytes. Hepatocytes (1×10^6 cells/ml) were incubated in Krebs-Henseleit bicarbonate buffer with 20 μM 4-methylumbelliferone, in the presence and absence of 5-pregnen-3β-ol-20-one sulfate (pregnenolone sulfate), an inhibitor of microsomal arylsulfatase (see Results and Ref. 19). Samples (0.5 ml) were collected at various times and centrifuged at 15,000 × *g* for 1 min. Free 4-methylumbelliferone and glucuronide in the supernatant (0.5 μl) were measured fluorometrically, as described above.

Sulfatase assay. 4-Methylumbelliferyl sulfate was stored as a 25 mM stock solution in 50 mM sodium acetate, pH 5.0, at −20°. Hepatocyte homogenates (0.4 mg of protein/ml of reaction mixture) or microsomes (50 μg of protein/ml of reaction mixture) were incubated with 5 μM 4-methylumbelliferyl sulfate at 37° in 50 mM HEPES, pH 7.4, containing 5 mM $MgCl_2$, 1 mM dithiothreitol, and 0.02% bovine serum albumin. Samples (10 μl) were removed at various time intervals and transferred to 1 ml of 0.02 M sodium carbonate buffer, pH 10.0, for the fluorometric determination of free 4-methylumbelliferone.

Hydrolysis and resynthesis of 4-methylumbelliferyl sulfate in the presence of microsomes and cytosol. Microsomes (12 μg of protein/ml of reaction mixture) and cytosol (150 μg of protein/ml of reaction mixture) were incubated together at 25° in 1 ml of the buffer described above for the assay of sulfatase. Hydrolysis of 4-methylumbelliferyl sulfate was monitored continuously from the fluorescence of liberated 4-methylumbelliferone. At various times indicated in Fig. 6, PAPS was added (final concentration, 0.5 μM) to allow resynthesis of the sulfate conjugate.

Results

Uptake and hydrolysis of sulfate and glucuronide conjugates of 4-methylumbelliferone by isolated hepatocytes. Sulfate and glucuronide conjugates of methylumbelliferone were taken up and hydrolyzed by intact hepatocytes; however, the amount of methylumbelliferone generated from the sulfate conjugate was at least 10-fold greater than that observed with the glucuronide conjugate (Fig. 1). The appearance of fluorescence reflects net free methylumbelliferone produced after hydrolysis and reconjugation. The finding that rates of 4-methylumbelliferone formation were consistently higher in homogenates than in intact cells (Fig. 2) and were increased by permeabilization of cells with saponin (Table 1) may be explained by either removal of a plasma membrane barrier or impaired resynthesis of the conjugate. The presence of a plasma membrane barrier for glucuronide and sulfate conjugates of methylumbelliferone has been suggested by recent studies in the perfused liver (20, 21). Rates of production of methylumbelliferone from the glucuronide conjugate remained about 10-fold lower than values observed with the sulfate conjugate in permeabilized cells (1.68 versus 17.5 pmol/min/10^6 cells). These differences in rates of fluorescent product generation from the two conjugates suggest that the transport and intracellular processing of the 4-methylumbelliferyl glucuronide and sulfate differ markedly in intact hepatocyes.

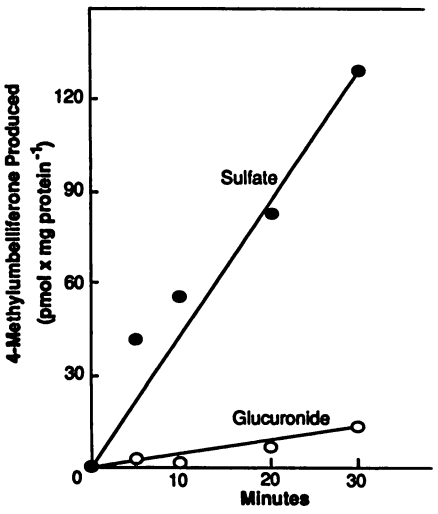

Fig. 1. Uptake and hydrolysis of 4-methylumbelliferyl sulfate and glucuronide by isolated hepatocytes. Hepatocytes (1 × 10^6 cells/ml) were incubated in Krebs-Henseleit bicarbonate buffer at 37° in the presence of either 5 μM 4-methylumbelliferyl sulfate or glucuronide. At various intervals, aliquots (10 μl) of the incubation medium were transferred to 1 ml of 0.02 M sodium carbonate buffer, and the fluorescence of 4-methylumbelliferone was measured as described in Materials and Methods. Each *point* is the average of duplicate samples.

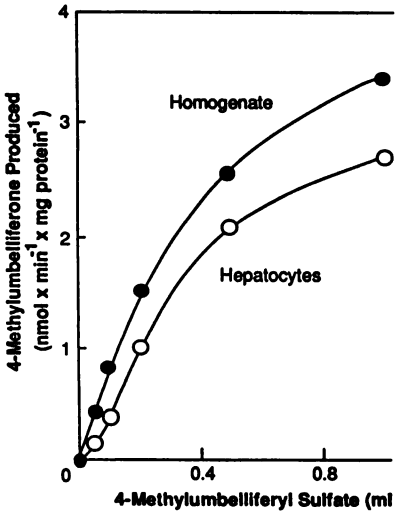

Fig. 2. Hydrolysis of 4-methylumbelliferyl sulfate as a function of substrate concentration in isolated hepatocytes and homogenates. Hepatocytes and homogenates of hepatocytes were prepared as described in Materials and Methods and incubated at a final protein concentration of 1 mg/ml. Rates of hydrolysis of 4-methylumbelliferyl sulfate at each substrate concentration tested were linear for at least 30 min. Values are averages of duplicate determinations of rates calculated from samples taken at four time intervals (0, 4, 15, and 30 min).

Formation of methylumbelliferone from 4-methylumbelliferyl sulfate was saturable in homogenates and whole cells (Fig. 2); however, rates of product formation as a function of substrate concentration were sigmoidal in intact cells and hyperbolic in homogenates. More complex kinetics in intact cells may be related to events influencing transport of the sulfate conjugate across the plasma membrane, as well as to the net effect of hydrolysis and resynthesis. Maximal rates of fluorescent product formation were higher in homogenates than in intact cells, and concentrations of 4-methylumbelliferyl sulfate required for half-maximal rates were lower in homogenates than in hepatocytes (Table 2). Because rates are most likely affected not only by synthesis and deconjugation reactions (see Discussion) but also by uptake and export processes, these half-maximal values represent only estimates of the concentrations required for half-maximal synthesis (i.e., K_m values).

TABLE 1

Effect of saponin on hydrolysis of 4-methylumbelliferyl sulfate by hepatocytes

Each experiment was performed with a fresh preparation of hepatocytes. Incubations and analyses were performed as described in Materials and Methods, using 5 μM 4-methylumbelliferyl sulfate as substrate. Cells treated with saponin were preincubated for 5 min with 75 μg of saponin/ml, centrifuged, and resuspended in fresh medium before addition of substrate. Values are averages ± standard errors of the number of experiments indicated in parentheses. Data were analyzed by analysis of variance.

Addition	Free methylumbelliferone	Glucuronide conjugate	Total methylumbelliferone
	pmol/min/10^6 cells		
None (7)	4.02 ± 0.49	7.46 ± 1.19	11.54 ± 1.50
Saponin (4)	17.48 ± 1.55[a]		17.48 ± 1.55[a]

[a] $p < 0.05$ (treated versus no addition).

Effect of various inhibitors on 4-methylumbelliferyl sulfate hydrolysis by intact cells and homogenates. Uptake and intracellular processing of 4-methylumbelliferyl sulfate, i.e., net hydrolysis and resynthesis of the conjugate, could be inhibited selectively by inorganic salts and organic sulfates. Sodium sulfite inhibited the hydrolysis of 4-methylumbelliferyl sulfate in a dose-dependent manner in both intact hepatocytes and microsomes (Fig. 3B); half-maximal inhibition occurred with about 100 μM Na_2SO_3. In contrast, sodium sulfate failed to inhibit hydrolysis in microsomes (Fig. 3A) but did inhibit uptake and processing of the conjugate by intact cells (half-maximal inhibition at 100 μM). Data summarizing experiments characterizing inhibition of 4-methylumbelliferyl sulfate hydrolysis in hepatocytes and homogenates by the two inorganic salts are presented in Table 2. Sodium sulfite increased concentrations of 4-methylumbelliferyl sulfate required for half-maximal rates of hydrolysis about 4-fold in intact cells and nearly 8-fold in homogenates (Table 2). Sodium sulfate also increased concentrations of substrate required for half-maximal rates in hepatocytes about 2-fold but did not inhibit hydrolysis of the conjugate in isolated microsomes. Thus, these inorganic anions appear to act by different mechanisms in whole cells and broken cell preparations. One likely explanation is that they compete for 4-methylumbelliferyl sulfate at the plasma membrane. Alternatively, sodium sulfate may enhance the formation of PAPS in intact cells, which would increase resulfation of 4-methylumbelliferone, thereby decreasing net fluorescent product generation.

The sulfate conjugates of cholesterol, pregnenolone, and estrone all inhibited hydrolysis of 4-methylumbelliferyl sulfate by microsomes (Fig. 3D). Half-maximal inhibition of hydrolysis of 4-methylumbelliferyl sulfate by homogenates occurred with about 1 μM pregnenolone sulfate and 10 μM estrone sulfate or cholesterol sulfate. Pregnenolone sulfate and estrone sulfate were also potent inhibitors of the generation of fluorescent product from 4-methylumbelliferyl sulfate in hepatocytes, with half-maximal inhibition also occurring with about 1 μM pregnenolone sulfate and about 10 μM estrone sulfate (Fig. 3C). In contrast, cholesterol sulfate did not inhibit 4-methylumbelliferyl sulfate hydrolysis by intact hepatocytes. Because it inhibited hydrolysis in homogenates but not in intact cells, the conclusion is that cholesterol sulfate was not taken up by intact

TABLE 2

Effect of various inhibitors on uptake and hydrolysis of 4-methylumbelliferyl sulfate by isolated hepatocytes and homogenates

Hepatocytes (1×10^6 cells/ml) or homogenates (1 mg/ml) were incubated with the concentrations of inhibitors indicated in the table. For each preparation, duplicate determinations of rates were made, as described in the legend to Fig. 2. Values used to calculate maximal rates and substrate concentration required for half-maximal rates are based on linear rates of product formation determined from five or six samples collected between 5 and 30 min. Incubations were performed as described in Materials and Methods, with six different substrate concentrations varying from 0.05 to 2 mM. Maximal rates and substrate concentrations required for half-maximal rates were determined using the weighted regression analysis of Wilkinson (24). Values are averages ± standard errors of the number of preparations indicated in parentheses. Data were analyzed statistically by analysis of variance.

Addition	Hepatocytes		Homogenate	
	Maximal rate	[Substrate] required for half-maximal rate	Maximal rate	[Substrate] required for half-maximal rate
	nmol/min/mg of protein	*mM*	*nmol/min/mg of protein*	*mM*
None	3.83 ± 0.66	0.75 ± 0.06 (7)	6.45 ± 1.20[a]	0.542 ± 0.033 (5)[a]
Na_2SO_4, 5 mM	4.69 ± 1.46	1.34 ± 0.22 (4)[b]		
Na_2SO_3, 5 mM	3.77 ± 0.63	3.38 ± 1.13 (4)[b]	3.71 ± 1.24	4.21 ± 1.25 (3)[b]
Pregnenolone-3-sulfate, 200 μM	0.66 ± 0.04	2.42 ± 0.825 (2)	0.24 ± 0.03	0.845 ± 0.012 (2)
Esterone-3-sulfate, 200 μM	1.35 ± 0.19	2.95 ± 0.99 (2)	0.45 ± 0.24	0.554 ± 0.294 (2)

[a] $p < 0.05$ (homogenate versus hepatocytes).
[b] $p < 0.05$ (addition versus none).

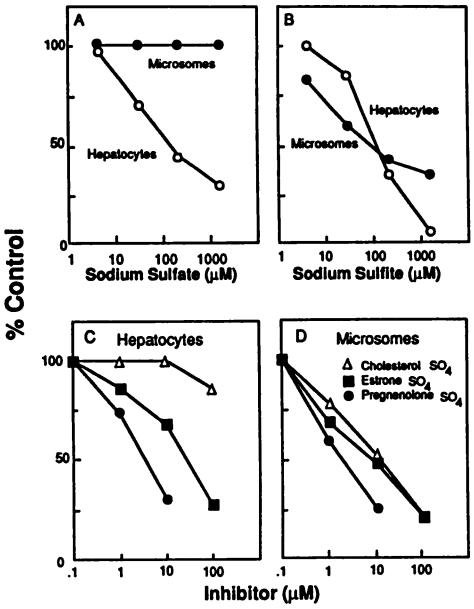

Fig. 3. Inhibition of 4-methylumbelliferyl sulfate hydrolysis in hepatocytes and isolated microsomes. A, Effect of sodium sulfate on hydrolysis of 4-methylumbelliferyl sulfate by hepatocytes (1×10^6 cells/ml) and microsomes (50 μg protein/ml). B, Effect of sodium sulfite on hydrolysis by hepatocytes and microsomes. C, Effect of steroid sulfates on hydrolysis by hepatocytes. D, Effect of steroid sulfates on hydrolysis by microsomes. Assays were performed using 20 μM 4-methylumbelliferyl sulfate as substrate. Rates of hydrolysis in the absence of added inhibitor were approximately 0.2 nmol/min/mg of protein for hepatocytes and 0.15 nmol/min/mg of protein for microsomes.

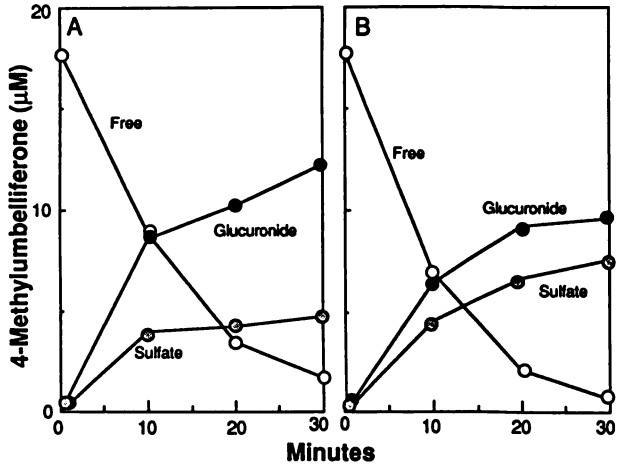

Fig. 4. Influences of pregnenolone sulfate on conjugation of 4-methylumbelliferone by isolated hepatocytes. Hepatocytes were incubated with 20 μM 4-methylumbelliferone, in the absence (A) and presence (B) of 100 μM pregnenolone sulfate, in Krebs-Henseleit bicarbonate buffer at 37°. Samples were withdrawn from the incubation medium at 0, 10, 20, and 30 min for the determination of free (○) 4-methylumbelliferone and the glucuronide (●) and sulfate (○) conjugates, as described in Materials and Methods.

cells (Fig. 3C). Both steroid sulfates elevated concentrations of 4-methylumbelliferyl sulfate required for half-maximal rates of hydrolysis about 4-fold in hepatocytes but not in homogenates (Table 2).

Effect of pregnenolone sulfate on conjugation of 4-methylumbelliferone by intact hepatocytes. In the absence of pregnenolone sulfate, glucuronide conjugates of 4-methylumbelliferone were produced at rates about twice those for sulfate conjugates (Fig. 4A). Addition of 100 μM pregnenolone sulfate, which inhibits sulfatase in intact hepatocytes, increased sulfate conjugate production and decreased glucuronide formation from 4-methylumbelliferone (Fig. 4B and Table 3). Glucuronide production decreased about 27% in the presence of pregnenolone sulfate. Simultaneously, sulfate conjugate production increased and essentially balanced the decrease in glucuronide production. Addition of pregnenolone sulfate increased the ratio of sulfate to glucuronide nearly 2-fold at 30 min of incubation, from 0.5 to 0.9.

Because conjugations of substrates to form sulfates and glucuronides represent competing pathways (22), inhibition of glucuronosyl transferase by pregnenolone sulfate might also explain the decreased glucuronide and increased sulfate conjugate production shown in Fig. 4 and Table 3. We, therefore, examined the effect of pregnenolone sulfate (100 μM) on glucuronosyl transferase in homogenates of rat hepatocytes incubated with methylumbelliferone (20 and 50 μM) and 0.2 mM UDP-glucuronic acid. Glucuronosyl transferase was assayed as described previously (6). With 50 μM methylumbelliferone, glucuronosyl transferase activity was 115 and 120 pmol/mg of protein/min in the presence and absence of pregnenolone sulfate, respectively. In the presence of 20 μM 4-methylumbelliferone, the rate of hydrolysis was approximately 50 pmol/mg of protein/min and was not altered by pregnenolone sulfate.

TABLE 3

Effect of pregnenolone sulfate on conjugation of 4-methylumbelliferone by isolated hepatocytes

Free 4-methylumbelliferone and metabolites of 4-methylumbelliferone generated by isolated hepatocytes were measured 30 min after incubation with 20 μM 4-methylumbelliferone as substrate. Incubations were performed with and without pregnenolone sulfate (100 μM), as described in Materials and Methods. Data are averages ± standard errors of three hepatocyte preparations. Data were analyzed statistically using a matched-pair *t* test.

Treatment	Free methylumbelliferone	Glucuronide conjugate	Sulfate conjugate	Sulfate to glucuronide ratio
		μM		
Control	2.92 ± 0.86	10.16 ± 1.14	4.91 ± 0.57	0.49 ± 0.10
Pregnenolone sulfate, 100 μM	3.04 ± 1.62	7.53 ± 1.29*	6.69 ± 1.22	0.91 ± 0.19

*$p < 0.05$ versus control.

Hydrolysis and reconjugation of 4-methylumbelliferyl sulfate in broken cell preparations. Hydrolysis and resynthesis via futile cycling of 4-methylumbelliferyl sulfate could be demonstrated in homogenates of liver by the addition of PAPS, the cofactor for sulfotransferase. Hydrolysis of 4-methylumbelliferyl sulfate by homogenates was linear in the absence of added PAPS for at least 30 min. Addition of PAPS decreased the formation of product in a dose-dependent manner (Fig. 5A). The departure from linear rates of 4-methylumbelliferone formation when PAPS (10–100 μM) was added is consistent with the hypothesis that hydrolysis and resynthesis of the sulfate conjugate occur.

Addition of cytosol to microsomes incubated with 100 μM PAPS reduced rates of hydrolysis of fluorescent product generation from methylumbelliferyl sulfate, in a complex manner (Fig. 5B). The lowest concentration of cytosol added to the reaction mixture (44 μg of protein) inhibited product formation completely; however, this inhibition lasted only 10 min and was followed by hydrolysis at rates comparable to those observed in the absence of added cytosol (Fig. 5B). The two higher concentrations of cytosol studied inhibited formation of free 4-methylumbelliferone completely for at least 30 min. In the absence of PAPS, large amounts of cytosol (Fig. 5A) did not inhibit formation of free 4-methylumbelliferone generated from the sulfate conjugate.

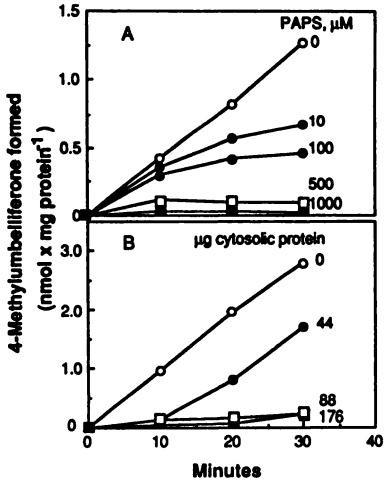

Fig. 5. Influence of PAPS (A) or cytosol (B) on hydrolysis of 4-methylumbelliferyl sulfate. Homogenates prepared from hepatocytes (0.4 mg of protein/ml of reaction mixture) or microsomes (50 μg of protein/ml) were incubated with 5 μM 4-methylumbelliferyl sulfate at 37° in 1 ml of reagent, as described in Materials and Methods. Samples (10 μl) were removed at various time intervals and transferred to 1 ml of 0.02 M sodium carbonate buffer, pH 10.0, for the fluorometric determination of free 4-methylumbelliferone. PAPS was added (A) at the concentrations indicated to reaction mixtures containing homogenates of isolated hepatocytes. Cytosol prepared from whole liver homogenates was added (B) to liver microsomes. Amounts of cytosol added are expressed as μg of protein of the 100,000 × *g* supernatant fraction added/ml of reaction mixture containing 100 μM PAPS. Each *point* is the average of duplicate samples and is expressed on the basis of the protein content of homogenate or microsomes. A typical experiment is shown.

Resynthesis of the sulfate conjugate when only 5 μM 4-methylumbelliferone was added to microsomes incubated with cytosol suggests that resynthesis of the sulfate conjugate occurs via a sulfotransferase with a relatively high affinity for 4-methylumbelliferone. At least four isoforms of sulfotransferase, with overlapping substrate specificities, have been reported for rat liver (23). To test for the presence of a transferase with a high affinity for methylumbelliferone, we measured rates of sulfation by cytosol prepared from hepatocytes as a function of 4-methylumbelliferone concentrations, ranging between 0.01 and 4 μM, in medium containing 100 μM PAPS. Analyses of these data by weighted regression analysis (24) indicated a K_m of 1.1 ± 0.2 μM for 4-methylumbelliferone and a V_{max} for hepatic sulfotransferase(s) of 3.03 ± 0.22 nmol/min/mg of cytosolic protein.

Futile cycling of 4-methylumbelliferyl sulfate and 4-methylumbelliferone in a reconstituted microsomal-cytosolic system. To test whether futile cycling via arylsulfatase and sulfotransferase could be demonstrated in a reconstituted system, the fluorescence generated by hydrolysis of 4-methylumbelliferyl sulfate by a mixture consisting of microsomes and cytosol was monitored continuously (Fig. 6). Rates of hydrolysis of 4-methylumbelliferone sulfate were linear until PAPS was added to the system, which enhanced removal of the fluorescent product (Fig. 6). Hydrolysis and resynthesis of the sulfate conjugate continued until PAPS was consumed, and fluorescence of free 4-methylumbelliferone then once again increased in a linear fashion. Repeated additions of PAPS inhibited production of fluorescent 4-methylumbelliferone, due to resynthesis of the sulfate conjugate. Following addition of 5 mM sodium sulfite, an inhibitor of microsomal 4-methylumbelliferone sulfatase (6), addition of PAPS caused a marked decrease in free 4-methylumbelliferone and reduced fluorescence to basal levels (Fig. 6). Based on rates of hydrolysis of 4-methylumbelliferyl sulfate and the time required for utilization of added PAPS, it was possible to calculate the amount of PAPS utilized to reconjugate 4-methylumbelliferone. Only about 10% of the added PAPS was utilized to reconjugate 4-methylumbelliferone liberated during hydrolysis of 4-methylumbelliferyl sulfate, suggesting that a large fraction of the added cofactor was hydrolyzed. In accord with this possibility, it is known that PAPS added to a 100,000 × *g* supernatant

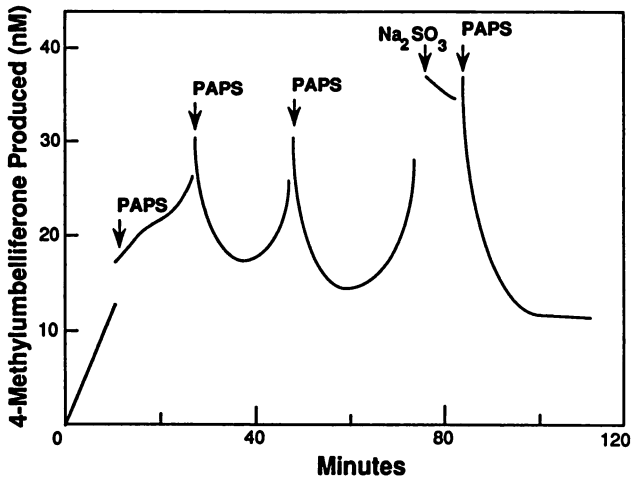

Fig. 6. Hydrolysis of 4-methylumbelliferyl sulfate in a reconstituted system. Microsomes (12 μg of protein) and cytosol (150 μg of protein) were incubated in 1 ml of buffer containing 5 μM 4-methylumbelliferyl sulfate, as described in Materials and Methods. At various intervals, as noted in the figure by *arrows*, PAPS (0.5 μM final concentration) or Na_2SO_4 (5 mM final concentration) was added. The breaks in the fluorescence record occurred when additions were made to the cuvette. The figure is a typical experiment illustrating a continuous recording of fluorescence (366 to 450 nm) at 25°.

fraction from brain is hydrolyzed rapidly to inorganic sulfate and 3′-phosphoadenosine-5′-phosphate (25).

Discussion

Uptake of 4-methylumbelliferyl sulfate by hepatocytes. Rates of uptake and net conversion of 4-methylumbelliferyl sulfate to methylumbelliferone by intact hepatocytes were about 10-fold greater than rates observed with the glucuronide conjugate, suggesting that transport of the sulfate conjugate across the hepatocyte plasma membrane and its hydrolysis within cells are more efficient than those of the glucuronide conjugate. Increased rates of hydrolysis of 4-methylumbelliferyl sulfate in homogenates (Fig. 2) and permeabilized cells (Table 2), compared with intact cells, imply that transport of sulfate conjugates across the plasma membrane is an important rate-limiting step in the metabolism of conjugates. Data in Fig. 1 suggest that rate limitation imposed by the plasma membrane is more pronounced under physiological conditions, where concentrations of sulfate conjugates in plasma are relatively low. For example, rates of hydrolysis product formation with 50 μM 4-methylumbelliferyl sulfate were nearly 3-fold higher in homogenates than in intact cells (153 versus 442 pmol/mg of protein/min), whereas rates of hydrolysis were only 30% higher in homogenates than in intact cells with 500 μM 4-methylumbelliferyl sulfate (Fig. 2). These data are in accord with recent reports indicating that the influx of sulfate and glucuronides of 4-methylumbelliferone into hepatocytes in perfused liver is limited by transport across the plasma membrane (20, 21). Although transport of conjugates across the plasma membrane is rate limiting, differences in rates of hydrolysis of sulfate and glucuronide conjugates of 4-methylumbelliferone in intact hepatocytes cannot be explained solely on the basis of differences in plasma membrane transport mechanisms. Permeabilization of hepatocytes with saponin increased rates of 4-methylumbelliferyl glucuronide hydrolysis about 4-fold; however, rates remained significantly lower than values observed with the sulfate conjugate. The interesting phenomenon of slower rates of hydrolysis of the glucuronide conjugate, compared with sulfate, may be explained, in part, by differences in access of conjugates to their respective hydrolases. For example, hepatic β-glucuronidase is compartmentalized within the lumen of the endoplasmic reticulum (26) or within lysosomes, whereas arylsulfatase is located on the cytosolic surface of the endoplasmic reticulum (19) and may have ready access to sulfate conjugates. Differences in protein binding of glucuronide and sulfate conjugates of 4-methylumbelliferone may also account for the observed differences in rates of hydrolysis of the two conjugates; however, the greater protein binding of sulfate conjugates, compared with glucuronides (22), argues against this possibility.

Inhibition of organic sulfate conjugate hydrolysis in hepatocytes. Hydrolysis of organic sulfate conjugates is inhibited by agents acting either on anion transport sites in the plasma membrane or on intracellular arylsulfatase. Sodium sulfate, which inhibited 4-methylumbelliferyl sulfate hydrolysis by hepatocytes but not homogenates, may either act at the plasma membrane or enhance resulfation by providing substrate for the formation of PAPS. Concentrations of 4-methylumbelliferyl sulfate required for half-maximal rates increased in the presence of sodium sulfate (Table 2), due possibly to competition for substrate binding sites at the plasma membrane. Further studies to test this possibility are warranted; however, it is noteworthy that a specific transporter for inorganic sulfate in the hepatocyte plasma membrane has been reported (27). Sodium sulfite, which inhibited hydrolysis in both homogenates and intact cells, most likely acts at both plasma membrane and intracellular sites of hydrolysis.

Cholesterol sulfate was a potent inhibitor of 4-methylumbelliferyl sulfate hydrolysis by isolated microsomes but not intact cells (Fig. 3). These observations argue strongly that cholesterol sulfate is not taken up into hepatocytes in quantities sufficient to inhibit arylsulfatase. Because the two steroid sulfates studied, pregnenolone sulfate and estrone sulfate, inhibited hydrolysis by both intact cells and broken cell preparations, it is not possible to judge whether they acted primarily at sites of hydrolysis by intracellular arylsulfatase or on uptake into cells. Because cholesterol sulfate inhibited hydrolysis of 4-methylumbelliferyl sulfate in microsomes but not homogenates (Fig. 5), it is concluded that this conjugate acts primarily on the hydrolytic enzyme. Inhibition of 4-methylumbelliferyl sulfate hydrolysis in intact hepatocytes by relatively low concentrations (<10 μM) of pregnenolone sulfate and estrone sulfate can be explained, in part, by competition for the small amounts of 4-methylumbelliferyl sulfate (20 μM) used in the experiments reported in Table 2 and Figs. 3 and 4. Low concentrations of substrate and inhibitors used in the studies described above approximate the low micromolar concentrations expected in the circulation under physiological conditions. Thus, arylsulfatase as well as plasma membrane transporters represent important sites of interaction between xenobiotics and endogenous compounds.

Arylsulfatase and futile cycling of sulfate conjugates. The hypothesis that sulfate conjugates undergo futile cycling via arylsulfatase and sulfotransferase is supported by data from intact cells as well as broken cell preparations. Arylsulfatase is present in the endoplasmic reticulum and is in close apposition to sulfotransferase in cytosol. Interaction between these two

activities was demonstrated when cytosol was added to microsomes (Fig. 5B). Addition of cytosol in the presence of PAPS decreased rates of hydrolysis of 4-methylumbelliferyl sulfate, by serving as a cofactor for resulfation. When increasing amounts of PAPS were added to a mixture of cytosol and microsomes, there was a dose-dependent decrease in the production of fluorescent product (Fig. 5A). These results are best explained by resynthesis of the sulfate conjugate. It is unlikely that PAPS inhibited arylsulfatase, because hydrolysis of 4-methylumbelliferyl sulfate was not inhibited by PAPS in the absence of sulfotransferase (Fig. 5B). Finally, addition of small amounts of PAPS to a reconstituted system of microsomes and cytosol resulted in futile cycling of the sulfate conjugate and free 4-methylumbelliferone, until PAPS was consumed and hydrolysis of the 4-methylumbelliferyl sulfate proceeded in the absence of reconjugation (Fig. 6). Conjugation of 4-methylumbelliferone at low concentrations and in the reconstituted system can be ascribed to the high affinity of sulfotransferase(s) for 4-methylumbelliferone (K_m about 1 μM; see Results).

Because it is difficult to extrapolate results from broken cell preparations to results in intact cells, we tested for futile cycling in isolated hepatocytes. The interaction between arylsulfatase and sulfotransferase in regulating sulfate conjugation in intact cells was demonstrated clearly by the actions of pregnenolone sulfate, which modified the profile of 4-methylumbelliferyl glucuronide and sulfate conjugates produced by hepatocytes (Fig. 4). When hydrolysis of 4-methylumbelliferyl sulfate via arylsulfatase was inhibited with pregnenolone sulfate, production of the sulfate conjugate was increased about 30% and was accompanied by a simultaneous decrease in 4-methylumbelliferyl glucuronide production of about 30%. Alterations in rates of sulfate conjugate production from 4-methylumbelliferone in intact cells, as well as in cell-free systems, by agents that inhibit arylsulfatase emphasize that arylsulfatase is indeed intimately involved in the regulation of sulfate conjugate production in hepatocytes.

In view of the importance of sulfation in the metabolism of endogenous compounds and the well established role for this process in the inactivation (28) and activation (29, 30) of carcinogens and mutagens, further studies on the regulation of arylsulfatase and sulfotransferase are needed. Futile cycling via synthesis and hydrolysis may well be an important determinant for the utilization as well as the production of important sulfate conjugates by the liver and other tissues.

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