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# Partial Characterization of the Steroidsulfatases in *Peptococcus niger* H4

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**The strictly anaerobic intestinal *Peptococcus niger* H4 synthesizes three different steroidsulfatase enzymes: a constitutive arylsulfatase and two inducible alkylsteroidsulfatases. The arylsulfatase desulfates estrogen-3-sulfates and phenylsulfates. The two alkylsteroidsulfatases desulfate, respectively, 3α-sulfates and 3β-sulfates of $\Delta^5$, 5α, and 5β androstanes, pregnanes, and bile acids. Cholesterol-3β-sulfate was not desulfated by the alkylsteroidsulfatases nor were steroids or bile acids that were sulfated in positions other than the 3 position. The alkylsteroidsulfatases were induced by their substrates; bile acid sulfates, however, were poor inducers of the 3β-sulfatase and did not induce the 3α-sulfatase activity. In intact bacterial cells, taurine and sulfite suppressed the induction of the alkylsteroidsulfatases and inhibited the activity of the arylsulfatase and alkylsteroidsulfatases. In cell homogenates, the arylsulfatase and alkylsteroidsulfatases activities were inhibited by sulfite and sulfate but not by taurine. Our results support the hypothesis that the main function of the steroidsulfatases in *P. niger* H4 is to provide the bacteria with sulfur for dissimilatory purposes.**

In previous publications, we reported the isolation and identification of several strictly anaerobic steroid- and bile acid-desulfating bacteria from the human and rat intestinal microflora (16, 24, 28, 30). Although comparative studies of fecal steroids and bile acids in germfree and conventional animals (12), as well as incubations of steroids and bile acids with fecal suspensions from rats and humans (3, 10), had indicated the presence of desulfating bacteria, steroid-desulfating bacteria have until recently not been isolated from the intestinal tract nor from any other source.

In the intestinal tract, bacterial desulfation is a prior condition to other bacterial modifications of steroids and bile acids (2, 22) and is known to stimulate the enterohepatic circulation of steroids and bile acids (9, 26, 31). Both experimental and clinical data support the hypothesis that changes in the intestinal bacterial sulfatase activity can affect steroid and bile acid reabsorption (11, 18, 21, 27, 29).

Bacterial sulfatase enzymes have been extensively studied (reviewed in references 7 and 8) except for the microbial steroid and bile acid sulfatases because pure cultures of steroidsulfatase- or bile acid sulfatase-producing organisms had not yet been isolated. Steroidsulfatases have been studied in molluscs (6, 25) and particularly in humans (5). The molluscan sulfatase enzymes desulfate arylsteroidsulfates (E1S) and 3β-sulfates of alkylsteroids with a $\Delta^5$ structure (DHEAS and cholesterolsulfate), and some also desulfate 3α5βAAS. In humans, the steroidsulfatases desulfate E1S and the 3β-sulfates of $\Delta^5$ alkylsteroids (DHEAS, 3β5PES, and cholesterolsulfate). The human steroidsulfatases play an important role in intermediary steroid metabolism, and their deficiency is associated with several clinical syndromes, e.g., X-linked ichthyosis (14). Human enzymes capable of hydrolyzing 3α-sulfates have never been reported.

In this report, we describe some properties of bacterial steroidsulfatases and bile acid sulfatases produced by the *Peptococcus niger* H4 strain that was isolated from the human intestinal tract (28). In substrate specificity studies, *P. niger* H4 desulfated 3α- and 3β-sulfates of alkylsteroids and bile acids with a $\Delta^5$, 5α, or 5β structure as well as arylsteroid-3-sulfates. It was suggested that several sulfatases with different substrate specificities were present in *P. niger* H4 (28). We now describe some properties of the sulfatase activity of *P. niger* H4 that support this suggestion. We examined the *P niger* H4 sulfatase activity in intact bacterial cells to study factors regulating sulfatase activity in near-physiological conditions and in crude cell extracts to establish whether the suggested presence of more than one sulfatase could be supported by differences in enzymatic properties. Our main purpose was not to define the enzyme characteristics—that obviously will need purified enzymes—but to obtain evidence for the coexistence of different enzymes.

## MATERIALS AND METHODS

**Abbreviations and trivial names.** The following abbreviations are used in this paper (trivial names appear in parentheses): E1S, 3-sulfate-1,3,5(10)-estratrien-17-one (estrone-3-sulfate); E2S, 3-sulfate-1,3,5(10)-estratrien-17β-ol (estradiol-3-sulfate); E3S, 3-sulfate-1,3,5(10)-estratrien-16α, 17β-diol (estriol-3-sulfate); pNCS, *p*-nitrocatecholsulfate; pNPS, *p*-nitrophenylsulfate; PdS, phenolphthaleindisulfate; 3α5αAAS, 3α-sulfate-5α-androstane-17-one (androsterone sulfate); 3α5βAAS, 3α-sulfate-5β-androstane-17-one; 3β5α AAS, 3β-sulfate-5α-androstane-17-one; 3β5βAAS, 3β-sulfate-5β-androstane-17-one; DHEAS, 3β-sulfate-5-androstene-17-one (dehydroepiandrosteronesulfate); 3β5αPAS, 3β-sulfate-5α-pregnane-20-one; 3β5PES, 3β-sulfate-5-pregnene-20-one (pregnenolonesulfate); aLCAS, 3α-sulfate-5α-lithocholic acid (allolithocholic acid sulfate); LCAS, 3α-sulfate-5β-lithocholic acid (lithocholic acid sulfate); iaLCAS, 3β-sulfate-5α-lithocholic acid (isoallolithocholic acid sulfate); iLCAS, 3β-sulfate-5β-lithocholic acid (isolithocholic acid sulfate); cholesterolsulfate, 3β-sulfate-5-cholestene. Nonsulfated steroids, bile acids, or phenols are abbre-

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viated as above but without "S," e.g., 3β5βAA, 3β-ol-5β-androstane-17-one.

**Culture conditions for *P. niger* H4 and preparation of resting cell suspensions and cell homogenates.** *P. niger* H4 was isolated from the human intestinal microflora. There was 100% DNA homology with the *P. niger* type strain (DSM 20475 or ATCC 27739), but the type strain possessed arylsulfatase and 3β-alkylsteroidsulfatase-bile acid sulfatase activity and no 3α-alkylsteroidsulfatases-bile acid sulfatase activity (28). *P. niger* H4 was routinely grown and maintained on Trypticase soy broth (TSB) (bioMérieux, Charbonniéres-les-Bains, France) supplemented with 8 mM taurine (2-aminoethanesulfonic acid).

Resting-cell suspensions of *P. niger* H4 were prepared as follows. Cells from late-exponential or early-stationary-growth-phase cultures were harvested anaerobically by centrifugation in airtight bottles (Nalgene, 500 ml; Nalge Co., Rochester, N.Y.) at 18,600 × *g* for 25 min at 4°C, washed twice, and resuspended in Tris hydrochloride buffer (0.05 M, pH 7.5)–0.077 M NaCl–0.05 M dithiothreitol (DTT) (Janssen Chimica, Beerse, Belgium) at a final cell protein concentration of 1.25 mg/ml as measured by the method of Lowry et al. (19). Resting cell suspensions were freshly prepared for every experiment.

To prepare cell homogenates, we freeze-pressed (X-25; A. B. Biotec, Stockholm, Sweden) stationary-growth-phase resting cell suspensions. Large cell wall fragments were removed by centrifugation for 15 min at 5,000 × *g* and 4°C and subsequent filtration through a sterile filter (0.22-μm pore size GS filter; Millipore S.A., Molsheim, France). The protein concentration of the cell homogenate was adjusted to a final value of 11 mg of protein per ml, and samples were stored at −70°C until used.

All manipulations and experiments except the freeze-pressing and centrifugation were done at 37°C in an anaerobic glove box (80% $N_2$, 10% $CO_2$, 10% $H_2$) of the type described by Aranki et al. (1).

**Standard assay conditions.** Desulfation of steroidsulfate or bile acid sulfate in growing cell cultures was assayed as follows. A 0.5-ml sample of a 3-day-old *P. niger* H4 culture on TSB plus 8 mM taurine with or without 0.13 mM steroidsulfate or 0.05 mM bile acid sulfate was used to inoculate 5 ml of TSB plus 8 mM taurine plus 0.13 mM steroidsulfate or 0.05 mM bile acid sulfate and incubated at 37°C under strictly anaerobic conditions. Unless otherwise indicated, cultures were analyzed after 3 days.

Standard assay conditions for resting cell suspensions were as follows: substrate concentration, 0.465 mM; bacterial protein, 125 μg/ml; [DTT], 2.5 mM; [Tris hydrochloride] (pH 7.3 for arylsulfates, pH 7.8 for alkylsteroidsulfates or bile acid sulfates), 0.05 M; incubation time, 60 min at 37°C under strictly anaerobic conditions; total assay volume, 1 ml. The reaction was terminated by the addition of 0.5 ml of 3 M sodium acetate (pH 5).

Standard assay conditions for cell homogenates were as follows. For arylsulfatase activity, the substrate concentration was 92 μM, bacterial protein was 1.57 mg/ml, [DTT] was 3.3 mM, [Tris hydrochloride] (pH 7) was 0.05 M, incubation time was 15 min at 37°C, and total assay volume was 1 ml. For 3β-alkylsteroidsulfatase and 3β-bile acid sulfatase activity, substrate concentration was 105 μM, bacterial protein was 1.57 mg/ml, [DTT] was 3.3 mM, [Tris hydrochloride] (pH 7.5) was 0.05 M, incubation time was 60 min at 37°C, and total assay volume was 1 ml. For 3α-alkylsteroidsulfatase and 3α-bile acid sulfatase activity, substrate concentration was 175 μM, bacterial protein was 3.14 mg/ml, [DTT] was 3.3 mM, [Tris hydrochloride] (pH 8) was 0.05 M, incubation time was 60 min at 37°C, and total assay volume was 1 ml.

Unless otherwise stated, we always used E1S to assay the arylsulfatase activity, DHEAS for the 3β-alkylsteroidsulfatase or 3β-bile acid sulfatase activity, and 3α5αAAS for the 3α-alkylsteroidsulfatase or 3α-bile acid sulfatase activity. The reactions were terminated by the addition of 0.5 ml of 3 M sodium acetate (pH 5).

**Products used.** The phenylsulfates pNPS and pNCS were from Aldrich Chemical Co. (Milwaukee, Wis.); PdS was supplied by EGA-Chemie (Steinheim, Federal Republic of Germany); pNC, phenolphthalein, and pNP were from Janssen Chimica (Beerse, Belgium).

Bile acids were from Steraloids Inc. (Wilton, N.H.) or Maybridge (Launceston, United Kingdom) or were prepared in our laboratory (12). Bile acid sulfates were all prepared in our laboratory (23).

Unconjugated steroids and steroidsulfates were obtained from Sigma Chemical Co. (St. Louis, Mo.) and Steraloids Inc. [6,7-$^3$H(N)]estronesulfate ammonium salt, [1,2,6,7-$^3$H(N)]DHEAS ammonium salt, and [9,11-$^3$H(N)]DHEA were from Dupont, NEN Research Products (Boston, Mass.). 3β5βAAS and [9,11-$^3$H(N)]androsteronesulfate were prepared by the same method as that used for bile acid sulfate synthesis (23).

**Analysis of sulfatase reaction products.** To determine the desulfation of pNPS, pNCS, and PdS, 5 ml of growing culture or 1 ml of the resting cell suspension assays was centrifuged for 15 min at 3,000 × *g*, and 3 and 0.8 ml of supernatant, respectively, were combined with equal volumes of 1 N NaOH. Absorption was measured spectrophotometrically at 420 nm for pNP, 515 nm for pNC, and 560 nm for phenolphthalein and compared with standard solutions. In growing cell cultures and in resting cell suspensions, desulfation products were analyzed by gas-liquid chromatography. In the cell homogenate experiments, sulfatase activity was studied with labeled steroidsulfates.

**(i) Gas-liquid chromatography.** Desulfation of steroidsulfate esters was studied by gas-liquid chromatography of nonderivatized or acetylated desulfated steroids in 3 ml of growing cultures or 1 ml of resting cell suspension assays as described previously (30).

To analyze the nondesulfated steroidsulfates, 3 ml of culture medium or 1 ml of resting cell suspension was desalted on a reversed-phase octadecylsilane bonded silica cartridge (J. T. Baker Chemical Co., Phillipsburg, N.Y.) and steroids were eluted with methanol. After the addition of 50 μg of 5α-cholestane, the eluate was evaporated and solvolyzed for 4 h at 37°C in 10 ml of methanol-acetone (1:9, vol/vol) acidified with 0.1 ml of 6 N HCl. The hydrolysate was neutralized with 0.5 ml of 25% $NH_4OH$, evaporated, and analyzed as described above. Bile acid sulfate hydrolysis was studied by gas-liquid chromatography as described in reference 24.

**(ii) Labeled products.** Labeled desulfation products in the cell homogenate assays were analyzed as follows. At the end of the experiment, 2 ml of diethyl ether was added to the assay mixtures and the tubes were vigorously shaken for 5 min and immediately stored at −20°C. After 4 h at −20°C, the liquid diethyl ether phase was poured off the frozen water phase and evaporated to dryness, and after the addition of 8 ml of Lumagel (Lumac Systems Inc., Titusville, Fla.), radioactivities were counted twice for 5 min in a Packard Tricarb 2660 Liquid Scintillation System (United Technologies, Packard, Warrenville, Ill.). Recovery of de-

TABLE 1. Induction of sulfatase activity with different steroidsulfates and phenylsulfates: substrate specificity of the induced sulfatase activity and efficacy of the inducers[a]

| Inducer | Activity with the following substrate: | | |
|---|---|---|---|
| | Aryl- and phenylsulfates | 3β-alkylsteroid- and bile acid sulfates | 3α-alkylsteroid- and bile acid sulfates |
| No inducer | + | − | − |
| Arylsteroid- and phenylsulfates | | | |
| Estrone-3-sulfate | ++ | − | − |
| Estradiol-3-sulfate | ++ | − | − |
| *p*-Nitrocatecholsulfate | ++ | − | − |
| *p*-Nitrophenylsulfate | ++ | − | − |
| Phenolphthaleindisulfate | ++ | − | − |
| 3β-alkylsteroid- and bile acid sulfates | | | |
| 3β-Sulfate-5α-androstane-17-one | +++ | +++ | − |
| 3β-Sulfate-5β-androstane-17-one | + | + | − |
| 3β-Sulfate-5-androstene-17-one | +++ | +++ | − |
| 3β-Sulfate-5α-pregnane-20-one | +++ | +++ | − |
| 3β-Sulfate-5-pregnene-20-one | +++ | +++ | − |
| 3β-Sulfate-5β-bile acids | + | + | − |
| 3β-Sulfate-5α-bile acids | + | + | − |
| 3β-Sulfate-$\Delta^5$-bile acids | + | + | − |
| 3α-alkylsteroid- and bile acid sulfates | | | |
| 3α-Sulfate-5α-androstane-17-one | ++ | ++ | +++ |
| 3α-Sulfate-5β-androstane-17-one | + | + | + |
| 3α-Sulfate-5α-bile acids | + | − | − (except aLCAS: +) |
| 3α-Sulfate-5β-bile acids | + | − | − (except LCAS: +) |
| Nonsulfated steroids | | | |
| Estrone | + | − | − |
| Dehydroepiandrosterone | + | − | − |
| 3α-ol-5α-Androstane-17-one | + | − | − |

[a] Sulfatase activity in resting cell suspensions from a 24-h TSB–8 mM taurine–0.13 mM steroidsulfate-, phenylsulfate-, or steroid-induced *P. niger* H4 culture expressed as a percentage of maximal desulfation rates. Standard assay conditions. +++, >75% of maximal desulfation rate; ++, 25 to 75% of maximal desulfation rate; +, <25% of maximal desulfation rate; −, no induction of sulfatase activity.

sulfated E1S, DHEAS, or 3α5αAAS was more than 95%; extraction of label in zero-time assays was less than 5%.

## RESULTS

**Sulfatase activity in intact *P. niger* H4 cells. (i) Optimal assay conditions.** Maximal 3α5αAAS and DHEAS desulfation (312 and 928 nmol/h/mg of protein, respectively) were obtained under standard assay conditions (see Materials and Methods). The E1S desulfation rate under standard assay conditions was 750 nmol/h/mg of protein.

DTT increased desulfation in anaerobic assays up to maximal rates at a DTT concentration of 2.5 mM. Aerobic incubation in the presence of DTT reduced sulfatase activity to 30% of that obtained in anaerobic conditions; aerobic incubation without DTT abolished sulfatase activity completely.

**(ii) Constitutive arylsulfatase activity versus inducible alkylsteroidsulfatase-bile acid sulfatase activity.** 3α- and 3β-alkylsteroidsulfatase activity was lost after two to three subcultures on alkylsteroid-free medium but was restored by the addition of 0.13 mM alkylsteroidsulfates to the culture medium. However, *P. niger* H4 retained its arylsulfatase activity on steroidsulfate-free media; the addition of 0.13 mM E1S and DHEAS nevertheless increased E1S desulfation from 420 to 750 and 1,900 nmol/h/mg of protein, respectively. Similar results were obtained in assays with cell homogenates.

**(iii) Time window for alkylsteroidsulfatase induction.** Resting cell suspensions prepared from cultures induced with 0.13 mM DHEAS for 4 h prior to harvesting at 4, 15, and 20 h after inoculation desulfated DHEAS at the normal rate (approximately 1,000 nmol/h/mg of protein). Resting cell suspensions from cultures induced for 4 h prior to harvesting at 28 and 52 h after inoculation did not desulfate DHEAS at all, indicating that alkylsteroidsulfatase induction was only possible during the first 20 h after inoculation (the exponential growth phase of the cultures). Resting cell suspensions prepared 16, 24, 48, and 168 h after inoculation of a *P. niger* H4 culture induced with 0.13 mM DHEAS at the time of inoculation all had comparable DHEAS desulfatase activity despite the fact that the DHEAS added as inducer was completely desulfated within 14 h after inoculation. We therefore assume that the alkylsteroidsulfatase, once induced, is only very slowly degraded.

**(iv) Induction specificity and induction efficacy of different steroidsulfates.** Noninduced *P. niger* H4 was grown on TSB plus 8 mM taurine supplemented with 0.13 mM of different steroids or steroidsulfates or 0.05 mM of bile acid sulfates, and the cells were harvested in the stationary growth phase. Addition of arylsulfates (estrogensulfates or phenylsulfates) enhanced the arylsulfatase activity compared with that in noninduced cells (Table 1). Supplementing the culture media with the 3β-sulfates of androstanes led, in all cases, to the induction of alkylsteroidsulfatase activity that was active on the 3β-sulfates of all the androstanes and of all pregnanes

TABLE 2. Factors interacting with steroidsulfatase induction and activity in intact *P. niger* H4 cells

| Compound tested (mM) | Effects on sulfatase expression in noninduced growing cells (interaction with sulfatase induction plus activity)[a] | | Effects on sulfatase activity in induced resting cells[b] | | Effects on sulfatase induction in noninduced resting cells[c] (% DHEAS desulfation rate[d]) |
|---|---|---|---|---|---|
| | % E1S desulfation rate[d] | % DHEAS desulfation rate[d] | % E1S desulfation rate[d] | % DHEAS desulfation rate[d] | |
| $SO_3^{2-}$ (5) | 45 ± 10 | 50 ± 10 | 36 ± 6 | 51 ± 4 | 73 ± 7 |
| $SO_3^{2-}$ (10) | | | 31 ± 8 | 34 ± 3 | 50 ± 6 |
| $SO_4^{2-}$ (5) | 100 ± 5 | 100 ± 2 | 100 ± 2 | 100 ± 3 | 100 ± 4 |
| $SO_4^{2-}$ (10) | | | 100 ± 2 | 100 ± 6 | 100 ± 10 |
| $NO_3^-$ (5) | 100 ± 7 | 100 ± 2 | 100 ± 2 | 100 ± 3 | |
| $PO_4^{3-}$ (5) | 100 ± 4 | 100 ± 4 | 100 ± 3 | 100 ± 1 | |
| Tyramine (5) | 140 ± 15 | 100 ± 7 | 155 ± 11 | 99 ± 4 | |
| Taurine (5) | 35 ± 4 | 60 ± 7 | 90 ± 3 | 83 ± 7 | 70 ± 3 |
| Taurine (10) | | | 66 ± 4 | 65 ± 6 | 43 ± 6 |
| Pyruvate (10) | | | 106 ± 4 | 150 ± 4 | 100 ± 6 |
| Pyruvate (20) | | | 112 ± 5 | 229 ± 25 | |

[a] E1S and DHEAS desulfation rates in noninduced growing cell cultures. Incubation times were 2, 4, and 6 h. The desulfation rates were corrected for differences in cell growth.

[b] E1S and DHEAS desulfation rates in resting cell suspensions collected from stationary-growth-phase cultures. Standard assay conditions except for 30-min incubation time.

[c] Resting cell suspensions from exponential-growth-phase cultures were induced for 4 h with 0.13 mM DHEAS in TSB supplemented with the compound tested prior to assaying for DHEAS sulfatase activity. Standard assay conditions.

[d] Desulfation rate expressed as percentage of desulfation rate in control cells ± SD ($n$ = 3). Control cells were submitted to the same pretreatment as the cells in the respective columns.

and bile acids tested. The 3α-sulfates of both the androstanes and the bile acids (3α-sulfates of pregnanes were not available) were not desulfated. Results identical to those found for cells induced with the 3β-sulfates of androstanes were also obtained for cells induced with the 3β-sulfates of $\Delta^5$PE (3β-ol-5-pregnene-20-one), 5α-PA (3β-ol-5α-pregnane-20-one), and 5β or 5α or $\Delta^5$ bile acids. Cells grown on media supplemented with either 3α5αAAS or 3α5βAAS were capable of desulfating all alkylsteroidsulfates tested, i.e., the 3β-sulfates as well as the 3α-sulfates of all androstanes, pregnanes, and bile acids tested. These results were confirmed by the observation that cell homogenates from cells grown on media supplemented with 0.13 mM DHEAS desulfated DHEAS but not 3α5αAAS, whereas cell homogenates from cells induced with 3α5αAAS were active on both DHEAS and 3α5αAAS. The addition of nonsulfated steroids to the culture media did not induce any sulfatase activity.

To investigate the induction efficiency of steroidsulfates, we determined the E1S, DHEAS, and 3α5αAAS desulfation rates of resting cell suspensions induced with different steroidsulfates (Table 1). 3β-Alkylsulfatase activity was most efficiently induced by DHEAS and 3β5αAAS, and 3α-alkylsulfatase was best induced by 3α5αAAS. The bile acids were inefficient inducers.

**(v) Physiological control of sulfatase induction and activity in intact *P. niger* H4 cells.** In growing cell cultures on TSB plus 0.13 mM E1S or DHEAS, we found that addition of sulfite or taurine suppressed E1S and DHEAS sulfatase activity; tyramine stimulated E1S sulfatase activity; and sulfate, nitrate, or phosphate had no effect on E1S or DHEAS sulfatase activity (Table 2). To determine whether the effects of these supplements were due to inhibition of enzyme induction and/or suppression of enzyme activity, we prepared resting cell suspensions from stationary-growth-phase cultures that had been induced with either 0.13 mM E1S or 0.13 mM DHEAS. Changes in the desulfating activity in these resting cell suspensions could be expected to be predominantly due to a change in enzyme activity and not to an effect on enzyme induction. The sulfatase activity of the resting cell suspensions was measured with either 0.465 mM E1S or 0.465 mM DHEAS plus the compounds to be tested in the standard Tris hydrochloride-NaCl-DTT buffer for short periods (30 min) only to minimize any effect of additional induction. Sulfite and taurine inhibited both the aryl- and alkylsteroidsulfatase activity (Table 2). Tyramine stimulated only the arylsulfatase activity, and pyruvate stimulated only the alkylsteroidsulfatase activity.

Modulation of the sulfatase induction was studied by preparing resting cell suspensions from noninduced late-exponential-growth-phase cultures. The resting cell suspensions were incubated for 4 h in TSB medium supplemented with 0.13 mM DHEAS plus the compounds to be tested. After the induction period, the cells were again harvested, washed, and resuspended in the Tris hydrochloride-NaCl-DTT buffer and assayed for DHEAS desulfation under standard conditions. Because differences in cell growth during the induction period could bias subsequent desulfation rates, we measured the cell protein concentration of the different resting cell suspensions after induction. No significant differences were found. Sulfite and taurine suppressed alkylsteroidsulfatase induction; sulfate or pyruvate apparently had no influence on the alkylsteroidsulfatase induction (Table 2).

**Desulfating activity in cell homogenates. (i) Influence of protein concentration and incubation time.** The formation rates of E1 and DHEA were linear with incubation time and protein concentration under standard conditions. The 3α5αAAS desulfation rate under standard conditions was linear for 30 min. Because 3α5αAAS transformations after 30 min under standard conditions were near the detection limit and 60-min incubations gave only 15% lower values than the predicted value for 60-min linear transformations, we routinely incubated 3α5αAAS for 60 min. This nevertheless implies that our 3α5αAAS sulfatase activity ($V_{max}$) is slightly underestimated.

**(ii) Optimum pH.** Maximal E1S sulfatase activity was obtained at pH 6.8 in 0.05 M Tris hydrochloride or at pH 7.5 in 0.05 M phosphate buffer. At the optimum pH of 6.5 in 0.05

M imidazole hydrochloride buffer, E1S sulfatase activity was only 75% of the maximal activity in Tris hydrochloride. DHEAS sulfatase activity was maximal at an optimum pH of 7.5 in 0.05 M Tris hydrochloride. Lower activities were obtained in 0.05 M imidazole hydrochloride buffer (70% of maximal activity at pH 7.1) and 0.05 M phosphate buffer (25% of maximal activity at pH 7.6).

For 3α5αAAS, maximal sulfatase activity was also obtained in 0.05 M Tris hydrochloride at pH 8. In the 0.05 M imidazole hydrochloride buffer, the optimum pH was 7.5 or higher (pH 7.5 being the upper buffering limit for this buffer system). In the 0.05 M phosphate buffer, the 3α5αAAS sulfatase activity was reduced to 30 to 40% of its maximal activity, but no optimum pH was discernible.

**(iii) Temperature activity curve and heat inactivation curve.** The optimum incubation temperature for both E1S sulfatase and DHEAS sulfatase under standard assay conditions was 37°C, and that for 3α5αAAS sulfatase was 40°C. A rapid decline in the activities of all three sulfatases was noted at higher temperatures.

To assess thermal inactivation, cell protein solutions were, prior to incubation, heated at 41, 51, or 61°C for 5, 10, or 20 min. The sulfatase activity toward all three substrates decreased only slightly after 5 min of preheating at 41°C. Heating at 61°C for only 5 min decreased the sulfatase activity for all three substrates to less than 25%.

The data collected during periods of exponential decline in activity were studied in terms of the rate constant $-K_t = \ln(A_t/A_0)$ for a first-order inactivation, where $A_0$ is the activity of the control, $A_t$ is the activity of the enzyme after preheating for a given time ($t$) at a certain temperature, and $K$ is the inactivation rate constant expressed per minute. The rate constants for inactivation were 0.020 for the E1S sulfatase versus 0.016 for the DHEAS sulfatase and 0.006 for the 3α5αAAS sulfatase at 41°C; at 51°C the values obtained were 0.22 for the E1S sulfatase and 0.11 for the DHEAS sulfatase versus 0.052 for the 3α5αAAS sulfatase.

**(iv) Effect of increasing substrate concentrations.** Lineweaver-Burk plots indicated that the Michaelis-Menten constants ($K_m$) were 643, 385, and 180 μM for E1S sulfatase, DHEAS sulfatase, and 3α5αAAS sulfatase, respectively. Substrate inhibition apparently occurred for the DHEAS sulfatase at DHEAS concentrations over 200 μM (Fig. 1). The maximum velocity values ($V_{max}$) as apparent from the Lineweaver-Burk plots were 22, 2.5, and 0.017 nmol/min/mg of protein for the E1S sulfatase, DHEAS sulfatase, and 3α5αAAS sulfatase, respectively. The 3α5αAAS sulfatase $V_{max}$ value is probably underestimated, as already mentioned.

**(v) Aerobic versus anaerobic assay conditions and effect of DTT concentrations.** The E1S sulfatase activity was equal in aerobic and anaerobic conditions and was not affected by DTT (Fig. 2). Maximal DHEAS sulfatase activity was obtained with 3.3 mM DTT in anaerobic conditions. In aerobically incubated preparations, DHEAS sulfatase activity was completely absent at DTT concentrations of 2.2 mM and lower; aerobic sulfatase activity in the presence of 3.3 mM DTT was only 15% of the maximal sulfatase activity obtained under anaerobic conditions. The addition of cysteine or β-mercaptoethanol as reducing agents gave equal DHEAS sulfatase activity, as did the addition of equimolar amounts of DTT. No measurable desulfation of 3α5αAAS could be induced by DTT in aerobic conditions. In anaerobic conditions, increasing DTT concentrations led to higher 3α5αAAS desulfation rates; maximal sulfatase activity for standard incubation conditions was reached at 5 mM DTT.

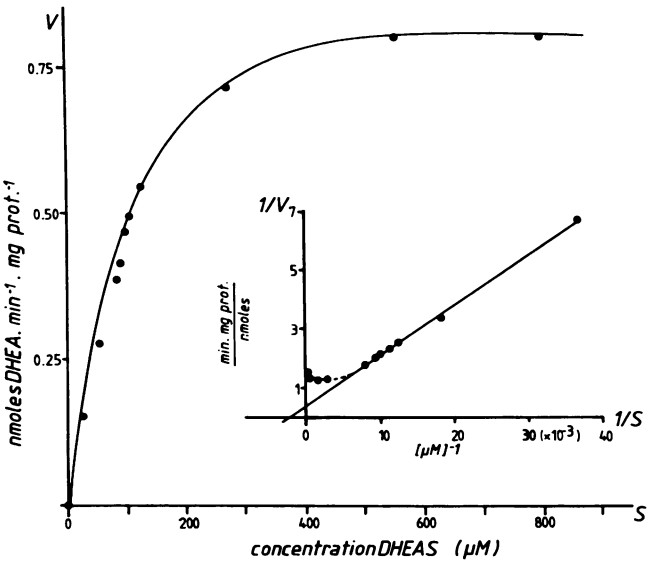


FIG. 1. Relationship between *P. niger* H4 DHEAS sulfatase activity and substrate concentration. Inset: Lineweaver-Burk plot. Standard assay conditions.

**(vi) Influence of steroidsulfates, phenylsulfates, nonsulfated steroids, and inorganic salts on E1S, DHEAS, and 3α5αAAS sulfatase activity.** The effect of other steroidsulfates or phenylsulfates on the desulfation of E1S, DHEAS, or 3α5αAAS was tested under the following conditions: [E1S], 60, 92, and 119 μM; [DHEAS], 69, 105, and 137 μM; [3α5αAAS], 156, 175, and 194 μM. At each of these substrate levels, three different concentrations of inhibitor (plus zero concentration) were tested. To test the effects of nonsulfated steroids or inorganic salts on the E1S, DHEAS, or 3α5αAAS sulfatase activity, only one concentration of E1S, DHEAS, or 3α5αAAS was used, i.e., the standard assay concentration.

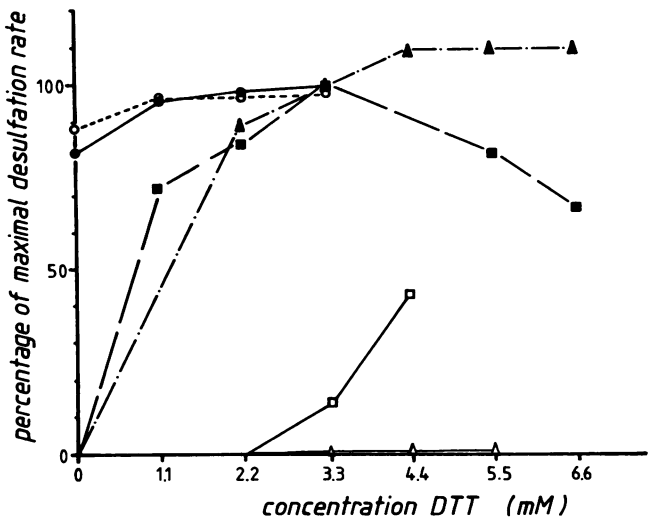


FIG. 2. Influence of DTT concentration on *P. niger* H4 E1S (●, ○), DHEAS (■, □), and 3α5αAAS (▲, △) sulfatase activity in cell homogenates in aerobic and anaerobic standard assay conditions. Open symbols, Aerobic conditions; closed symbols, anaerobic conditions.

TABLE 3. Effects of steroidsulfates, phenylsulfates, steroids, and inorganic salts on *P. niger* H4 cell homogenate E1S, DHEAS, and 3α5αAAS sulfatase activity[a]

| Compound added (mM) | E1S sulfatase | DHEAS sulfatase | 3α5αAAS sulfatase |
|---|---|---|---|
| Estrone-3-sulfate | — | — | — |
| Estradiol-3-sulfate | CI | — | — |
| Estriol-3-sulfate | CI | — | — |
| *p*-Nitrocatecholsulfate | CI | — | — |
| *p*-Nitrophenylsulfate | CI | — | — |
| Phenolphthaleindisulfate | CI | — | — |
| 3α-Sulfate-5α-androstane-17-one | NI | NCI | — |
| 3α-Sulfate-5β-androstane-17-one | — | NI | CI |
| Lithocholic acid-3-sulfate | — | NI | — |
| 3β-Sulfate-5α-androstane-17-one | — | CI | — |
| 3β-Sulfate-5β-androstane-17-one | — | CI | — |
| Dehydroepiandrosteronesulfate | NI | — | NI |
| 3β-Sulfate-5-pregnene-20-one | — | CI | — |
| Isoallolithocholic acid-3-sulfate | — | CI | — |
| Isolithocholic acid-3-sulfate | — | CI | — |
| Estrone | NI | NI | — |
| Dehydroepiandrosterone | NI | I | — |
| 3α-ol-5α-Androsterone-17-one | NI | NI | — |
| 3β-ol-5-Pregnene-20-one | — | I | — |
| $CN^-$ (5) | NI | I (52) | — |
| $F^-$ (5) | NI | I (74) | — |
| $SO_3^{2-}$ (10) | (I) (93) | I (76) | I (72) |
| $SO_4^{2-}$ (10) | (I) (96) | I (74) | I (82) |
| $NO_2^-$ (10) | NI | (I) (92) | — |
| $NO_3^-$ (10) | NI | NI | — |
| $PO_4^{3-}$ (10) | NI | NI | — |
| Taurine (10) | NI | NI | NI |

[a] I, Inhibition; (I), weak inhibition; NI, no inhibition; CI, competitive inhibition; NCI, noncompetitive inhibition; —, not tested. In parentheses are shown the percentage of residual activity of the sulfatase enzyme for 92 μM E1S, 105 μM DHEAS, and 175 μM 3α5αAAS and the concentration of inorganic salt indicated. Assay conditions are as described in Materials and Methods.

The concentrations of the nonsulfated steroids and inorganic salts are given below. The assay conditions were otherwise as described for the standard assay.

**E1S sulfatase activity.** E1S desulfation was inhibited by the addition of E2S, E3S, and the phenylsulfates PdS, pNCS, and pNPS (Table 3). The reciprocal plots were all compatible with competitive inhibition. E3S was a weaker inhibitor (15% reduction of E1S desulfation at equal concentrations of E1S and E3S) than E2S (50% reduction at equal concentrations). Of the phenylsulfates, pNPS was the strongest inhibitor and PdS the weakest (70, 50, and 15% reduction of E1S desulfation with equal concentrations [92 μM] of inhibitor and substrate for, respectively, pNPS, pNCS, and PdS). DHEAS and 3α5αAAS did not inhibit the E1S sulfatase activity, nor did 92 or 141 μM of the nonsulfated E1, DHEA, or 3α5αAA have any effect on E1S sulfatase activity. For $SO_3^{2-}$ and $SO_4^{2-}$, a small inhibitory effect was observed. Taurine (1, 5, or 10 mM) did not have any effect on the E1S sulfatase activity.

**DHEAS sulfatase activity.** DHEAS desulfation was inhibited by 3β5αAAS, 3β5βAAS, 3α5αAAS, 3β5PES, iaLCAS, and iLCAS (Table 3). The reciprocal plots of the inhibition experiments with 3β5αAAS, 3β5βAAS, 3β5PES, iaLCAS, and iLCAS were compatible with competitive inhibition. The reciprocal plot of the inhibition experiment with 3α5αAAS was compatible with noncompetitive inhibition. 3α5βAAS, LCAS, and cholesterolsulfate did not inhibit the DHEAS sulfatase activity. For equimolar amounts of substrate and inhibitor (105 μM), we found that 3β5βAAS and iLCAS both reduced the DHEAS sulfatase activity by 12%, iaLCAS and 3β5PES reduced it by approximately 25%, and 3β5αAAS reduced it by 30%. For nonsulfated steroids, 92, 141, or 184 μM of E1 or 3α5αAA did not inhibit DHEAS sulfatase activity; 69, 105, and 137 μM of DHEA or 3β5PE reduced the activity by, respectively, 10, 25, and 30%. Concentrations of 1 to 50 mM of inorganic salts were tested for their effect on DHEAS sulfatase. $NO_3^-$ had no effect at all; $SO_3^{2-}$, $SO_4^{2-}$, $NO_2^-$, $CN^-$, and $F^-$ inhibited DHEAS sulfatase; and $PO_4^{3-}$ also inhibited DHEAS sulfatase but only when added in high concentrations (20 or 50 mM). Taurine (1, 5, or 10 mM) had no effect on the DHEAS sulfatase activity.

**3α5αAAS sulfatase activity.** Inhibition of 3α5αAAS sulfatase activity was observed with 3α5βAAS but not with DHEAS (Table 3). The reciprocal plot showed the inhibition by 3α5βAAS to be compatible with competitive inhibition. The inorganic salts $SO_3^{2-}$ and $SO_4^{2-}$ (1, 5, and 10 mM) inhibited the 3α5αAAS sulfatase activity. Taurine (1, 5, and 10 mM) did not inhibit the 3α5αAAS sulfatase activity.

## DISCUSSION

The results presented in this report give evidence that the total steroidsulfatase activity of *P. niger* H4 is due to the existence of three different sulfatases, a constitutive arylsteroidsulfatase that also desulfates phenylsulfates and two substrate-induced alkylsteroidsulfatases, a 3α-sulfatase and a 3β-sulfatase.

First, the varying pH curves and buffer effects are compatible with differing ionization changes at the active site(s) of the enzymes since the pKs of the substrates are well below the pHs studied. Second, the E1S sulfatase was more sensitive to thermal inactivation than was the DHEAS sulfatase, which was, in turn, more sensitive than the 3α5αAAS sulfatase. Third, the E1S sulfatase activity was not sensitive to oxygen or to the presence of the reducing agent DTT, contrary to the DHEAS and 3α5αAAS sulfatases, which were very sensitive to these factors. Fourth, E1S sulfatase was not significantly inhibited by any of the inorganic salts tested, while DHEAS sulfatase and 3α5αAAS sulfatase were inhibited by various inorganic salts. And last, the presence or absence of inhibition of E1S, DHEAS, and 3α5αAAS desulfation by other steroidsulfates was clearly different for the three sulfatase activities. Arylsulfates (arylsteroidsulfates and phenylsulfates) inhibited the E1S sulfatase activity; 3β-alkylsteroidsulfates inhibited the DHEAS sulfatase activity but not the E1S sulfatase or 3α5αAAS sulfatase activity; and 3α-alkylsteroidsulfates inhibited the 3α5αAAS sulfatase but not the E1S sulfatase or the DHEAS sulfatase. The effect of arylsulfates such as E1S upon the DHEAS sulfatase or 3α5αAAS sulfatase activity could not be studied because all cells, uninduced cells as well as induced cells, possess high E1S sulfatase activity.

The constitutive nature of the arylsteroidsulfatase versus the substrate-induced character of the alkylsteroidsulfatases and the fact that 3β-sulfate alkylsteroids do not induce 3α-alkylsteroidsulfatase activity add further strength to the hypothesis that there are three different enzymes. The enhanced arylsteroidsulfatase activity after the addition of

steroidsulfates does not contradict its constitutive character. It is, however, surprising that alkylsteroidsulfates enhance the arylsteroidsulfatase activity and equally that 3α-alkylsteroidsulfates induce 3β-alkylsteroidsulfatase activity. Unequivocal proof of the existence of different steroidsulfatases can only be obtained after extensive purification and physical separation of the respective proteins. However, the irreversible inactivation of both the 3α- and 3β-alkylsteroidsulfatases in an aerobic environment (50% reduction of activity after 12 h at 4°C) poses a major obstacle to the purification of the *P. niger* H4 alkylsteroidsulfatases.

The apparent $K_m$ found for E1S sulfatase is of the same order of magnitude ($10^{-3}$ M) as the $K_m$s reported for other (aerobic) bacterial arylsulfatases, although these enzymes were assayed with phenylsulfates (7, 8). On the basis of their relative activities with phenylsulfates as substrates and their inhibition or lack of inhibition by cyanide, phosphate, or sulfate ions, these bacterial arylsulfatases have been subdivided into type 1 and type 2 enzymes (8). The *P. niger* H4 arylsulfatase together with other bacterial phenylsulfatases (8) fit into neither of these two groups. The *P. niger* H4 arylsteroidsulfatase differs considerably in some respects from the human E1S sulfatase. The bacterial $K_m$ (0.6 mM) is considerably higher than the human $K_m$ (1 μM); the bacterial sulfatase has a lower optimal temperature (37°C versus 55°C for the human sulfatase) and is much more sensitive to heating. The human arylsulfatase is inhibited by DHEAS and by unconjugated steroids, while the bacterial arylsulfatase is not. These differences in $K_m$s and inhibition characteristics between bacterial and human arylsulfatase probably reflect different metabolic roles.

It is still not clear whether the human steroidsulfatase that desulfates DHEAS and cholesterolsulfate is different from the human E1S sulfatase. The human steroidsulfatase, and also the steroidsulfatases found in molluscs, are specific for the 3β-sulfates of the $\Delta^5$ steroid series to which DHEAS and cholesterolsulfate belong. The 3β-alkylsteroidsulfatase from *P. niger* H4 is active not only on the 3β-sulfates of the $\Delta^5$ series but also on the 3β-sulfates of the 5α and 5β series of steroids. Contrary to the human steroidsulfatase, the *P. niger* H4 sulfatase does not desulfate cholesterolsulfate. A sulfatase enzyme active on 3α-sulfates of steroids, as in *P. niger* H4, has not yet been described in any other organism. Other differences between the human steroidsulfatase and the bacterial 3β-alkylsteroidsulfatase are the $K_m$s (367 μM versus 1 μM for the bacterial and human sulfatases, respectively, with DHEAS as the substrate) and the factors that affect the sulfatase activity. The human steroidsulfatase was strongly inhibited by unconjugated steroids (5), contrary to the bacterial 3β-alkylsteroidsulfatase, which was not strongly affected by unconjugated steroids but was sensitive to inorganic salts, especially sulfate and sulfite. Again, these differences between human and bacterial alkylsteroidsulfatase probably reflect the different metabolic functions of these enzymes.

In a previous publication, we suggested that the metabolic function of the *P. niger* H4 sulfatase activity was to provide sulfite that acted as an electron acceptor (30). The sulfonic acid group of taurine as well as sulfite, but not sulfate, is quickly and completely reduced to $H_2S$ by *P. niger* H4. The sulfur hydrolyzed from the steroidsulfates is also reduced to $H_2S$, although it is not clear how this is done because free sulfate is not reduced by *P. niger* H4. The fact that taurine and sulfite (i.e., sources of reducible sulfur) in non-growth-limiting concentrations inhibit sulfatase induction and activity is compatible with the hypothesis that these sulfatases function to provide the bacteria with an alterative source of reducible sulfur in the absence of other sources. For the bacterial sulfatases that have been studied until now, it was generally accepted that the availability of carbon rather than sulfur was the main regulating factor of sulfatase induction, especially alkylsulfatase induction (7, 8). Exceptions to that rule are a choline-desulfating enzyme (13) and a lithocholate sulfate-hydrolyzing enzyme (17), the induction of both of which is markedly inhibited by inorganic sulfate. Both of these enzymes were reported to function in the assimilatory acquisition of sulfur.

The tyramine-mediated enhancement of the arylsulfatase activity that we observed in *P. niger* H4 was also observed for the phenylsulfatase activity of *Pseudomonas* strain C12B (15). This effect was attributed to the formation of a complex between tyramine and the phenylsulfate that had a higher $K_m$ and $V_{max}$ than the phenylsulfate alone. Tyramine-mediated stimulation has also been observed for arylsulfotransferases (4). We also observed a stimulation of the alkylsteroidsulfatase activity by pyruvate. We suggest that the higher demand for electron acceptors that follows the bacterial metabolism of pyruvate stimulates sulfatase activity because this provides the bacteria with electron acceptors.

A comparison of the E1S, DHEAS, and 3α5αAAS sulfatase activities in intact resting cells and cell homogenates reveals some interesting differences. The results of the studies on the modulation of the sulfatase activity in resting cell suspensions must be interpreted with caution, however, because several factors, such as membrane transport or bacterial metabolism of the potential modulating compounds, may bias the results. The fact that taurine suppressed E1S and DHEAS sulfatase activity in intact cells but not in cell homogenates could be due to the degradation in intact cells of taurine to sulfite, which is possibly the real enzyme inhibitor. Other differences between intact cells and the cell homogenates, i.e., the absence of an inhibiting effect of sulfate on the 3β-alkylsteroidsulfatase in intact cells and the inhibition of the arylsulfatase by sulfite in intact cells as opposed to the effects of sulfate and sulfite on these enzyme activities in cell homogenates, cannot readily be explained from our experiments. Another interesting difference between intact and homogenized *P. niger* H4 cells is that for equal amounts of cell protein, the E1S sulfatase is less active than the DHEAS sulfatase in intact cells but almost ten times more active in cell homogenates. This could be compatible with a rate-limiting arylsteroid transport system in the bacterial cell membrane, as was reported for some bacteria (20), and/or with a cytosolic localization of the E1S sulfatase yielding higher recoveries during the cell homogenization process.

Substrate specificity studies of several other steroid- and bile acid-desulfating intestinal microorganisms that we recently isolated suggest the existence of several more steroidsulfatases with substrate specificities different from that of the *P. niger* H4 enzymes (30). The *P. niger* H4 enzymes are obviously only the first examples of what is apparently a diverse group of enzymes.

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