Effect of Sodium Sulfate on the Hydrolysis of 17-Hydroxycorticosteroid- and p-Nitrophenyl-glucurononides with β-Glucuronidase Preparations from Bovine Liver

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Previous papers dealt with the discovery that hydrolysis of 17-hydroxycorticosteroid glucuronides in urine with β-glucuronidase preparations from bovine liver is increased by adding sodium sulfate to the incubation medium. Here, we conclude that sodium sulfate not only increases the activity of bovine liver β-glucuronidase on 17-hydroxycorticosteroid glucurononides and p-nitrophenyl glucuronides, but also removes the inhibitory activity of substances of high molecular weight in urine and, moreover, inhibits urine putrefaction during the hydrolysis. The net effect is an increased yield of urinary 17-hydroxycorticosteroids. In the incubation with sodium sulfate (Na₂SO₄, final concentration 80 g/liter, 500 Fishman units of β-glucuronidase per milliliter of urine, pH 5.0, 48 °C, 18 h), the analytical recovery of 17-hydroxycorticosteroid glucuronides added to 12 urine samples proved to be 98 ± 1.8% (95–100).

The main difficulty in all known methods for determining urinary steroids is the hydrolysis of the steroid conjugates (1, 2).

Our previous papers (3–5) reported the increased hydrolysis of urinary 17-hydroxycorticosteroid glucuronides by β-glucuronidase (EC 3.2.1.31) preparations from bovine liver when sodium sulfate was added to the incubation medium. The purpose of the work described in this paper was to determine whether sodium sulfate (a) increases the enzyme activity for both 17-hydroxycorticosteroid glucuronides and nonsteroid glucuronides, (b) removes the inhibitory activity on the enzyme of high-molecular-weight substances in the urine, or (c) if other reactions occurring in the presence of sodium sulfate cause the increase of the hydrolysis, or if possibly the effect is explained by some combination of these three factors.

Procedures

Separation of 17-Hydroxycorticosteroid Glucuronides

One liter of urine was dialyzed at 5 °C for one day through a cellophane membrane against 10 liters of distilled water. The dialysate was applied to a 5 × 20 cm column containing Amberlite XAD-2. The column was washed with 500 ml of water. The steroid glucuronides were eluted from the resin with 500 ml of methanol. After removing the methanol under reduced pressure, we dissolved the residue in 10 ml of water, washed the solution three times with 30 ml of methylene chloride, and applied it to a 4 × 50 cm column containing Sephadex G-25. The steroid glucuronides were eluted with water, 20-ml fractions being collected. Fractions 18 to 20 contained the 17-hydroxycorticosteroid glucuronides (solvolyzable 17-hydroxycorticosteroids, the steroid sulfate, made up less than 5% of these fractions).

Separation of Inhibitors of β-Glucuronidase

Urine was centrifuged at 3000 rpm (1000 × g) for 20 min, and the supernatant fluid was applied to a Amberlite XAD-2 column as mentioned above. The adsorbed fraction was dialyzed against water. After heating at 60 °C for 30 min to inactivate urinary β-glucuronidase the high-molecular-weight (nondialyzable) fraction was used as β-glucuronidase inhibitor. In another experiment the supernatant fluid was directly dialyzed against water.

Assay of Enzyme Activity with 17-Hydroxycorticosteroid Glucuronides as Substrate

The reaction mixture, consisting of 4 ml of water, 0.5 ml of acetate buffer (0.67 mol/liter, pH in the range of 4 to 6), 0.5 ml of 17-hydroxycorticosteroid glucuronide solution (0.6 mmol/liter, as cortisol) and 0.1 ml of di-
Diluted β-glucuronidase solution (300 Fishman units), was incubated at 37 °C for 3 h in the presence of sodium sulfate (final concentration, 80 g/liter). After incubation, 0.5 g of sodium sulfate was added to the hydrolysate, followed by extraction with 30 ml of methylene chloride. The extract was successively washed with 3 ml of 0.1 mol/liter NaOH in 100 g/liter Na2SO4 and 3 ml of 100 g/liter Na2SO4. Ten milliliters of the methylene chloride extract was added to 1.0 ml of ethanol, followed by shaking with 1.5 ml of Porter–Silber reagent (1 mg of phenylhydrazine-HCl per milliliter of diluted sulfuric acid (concentrated H2SO4/H2O 64/36, by vol).1 For the sample blank, instead of Porter–Silber reagent, 1.5 ml of the diluted sulfuric acid was used. After removing the methylene chloride layer, we incubated the reagent layer at 60 °C for 30 min, the surface of the layer being kept 1 to 2 cm lower than that of the water bath. During the incubation the remaining methylene chloride evaporated. The absorbance of the sample vs. the blank was measured at 410 nm in 10 mm cuvettes (value A). Reagent blank, with water instead of 17-hydroxycorticosteroid glucuronide solution, was carried through the same procedure without β-glucuronidase (value B). Value B was subtracted from value A to obtain the true absorbance of the sample. Cortisol was used as a standard: 5.0 ml of the working standard solution (10 mg of cortisol per 1000 ml of water) plus 0.5 ml of acetate buffer without the enzyme was carried through the procedure, starting with methylene chloride extraction, in the presence of 1 g of sodium sulfate. This procedure is reported in detail in previous papers (3, 4).

We then conducted the same experiment, using a starting mixture without sodium sulfate, and added 1 g of Na2SO4 to the medium after incubation. The rest of the procedure was conducted as mentioned above.

In other assays with β-glucuronidase inhibitor, 4 ml of the inhibitor solution instead of water were used for incubation.

Assay of Enzyme Activity with p-Nitrophenyl Glucuronide as Substrate

The enzyme activity was measured according to a modification of Nobenago’s method (6). Reaction mixtures consisted of 0.1 ml of 40 mmol/liter p-nitrophenyl glucuronide, 0.3 ml of 0.67 mol/liter acetate buffer (pH in the range of 4 to 6), 0.5 ml of water or 0.5 ml of inhibitor solution, and 2.2 ml of water, and were incubated with 0.1 ml of diluted β-glucuronidase at 37 °C for 1.5 h in the absence or in the presence of sodium sulfate (final concentration, 80 g/liter). After incubation, 1 ml of 0.2 mol/liter NaOH was added to stop the reaction and the p-nitrophenol liberated was measured at 400 nm in a photometer.

In another assay, we used 0.1 ml of 10 mmol/liter phenolphthalein glucuronide instead of 0.1 ml of 40 mmol/liter p-nitrophenyl glucuronide and, after incubation (pH 5.2, 37 °C, 1 h), 1.5 ml of glycine buffer (pH

1 Diluted sulfuric acid: 64% (by vol) H2SO4 ("super special" grade), or 66% (by vol) H2SO4 (concentrated, analytical grade).
to that without sodium sulfate, for the enzyme preparations (Sigma, USA; Hokuiken Sapporo, Japan; Tokyo-zoki, Tokyo, Japan; Iatron, Tokyo, Japan) the figures were 1.31, 1.30, 1.54, and 1.66 for p-nitrophenyl glucuronidase; 1.35, 1.37, 1.38, and 1.44 for phenolphthalein glucuronidase; and 1.64, 1.80, 1.86, and 1.82 for 17-hydroxyxocorticosteroid glucuronides, respectively.

Effect of Sodium Sulfate on Inhibition of β-Glucuronidase by Urinary High-Molecular-Weight Substances

We used the above starting β-glucuronidase preparation as enzyme and p-nitrophenyl- and 17-hydroxyxocorticosteroid glucuronides as substrate. The inhibitor, the Amberlite XAD-2 treated urinary high-molecular-weight fraction, was prepared as described above.

As seen in Figures 2 and 3, in the absence of sodium sulfate the rate of hydrolysis with the β-glucuronidase preparation in the case of both substrates clearly decreased at each pH when the urinary inhibitor was added to the reaction system, but this inhibition was remarkably suppressed by addition of sodium sulfate, the rate of hydrolysis being very nearly the same as if no inhibitor were present.

When 15 urine samples that had only been dialyzed against water were used as inhibitor, the comparative rates of hydrolysis for 17-hydroxyxocorticosteroid glucuronides were as follows (incubation: 300 Fishman units of β-glucuronidase, pH 5.0, 37 °C, 3 h): with no sodium sulfate present, the values without and with the inhibitor were respectively 66 ± 3.6% (mean ± standard deviation) and 40 ± 7.4% of the value found without the inhibitor but with sodium sulfate present. The value with inhibitor present was 97 ± 2.1% when sodium sulfate was also present.

Figure 4 shows the effect of sodium sulfate and of the dialysis on the activities of urinary β-glucuronidase (substrate: p-nitrophenyl glucuronide, pH 4.6). Without sodium sulfate the values for urinary β-glucuronidase in the dialyzed fractions (designated as β-Glu) are lower than those in the corresponding original urines, while with sodium sulfate both values—those of dialyzed fractions (designated as β-Glu) and those of the original urines—are almost the same. At the same time the values with sodium sulfate present are always higher.

between 4.4 and 4.8. The activity of the starting enzyme preparation without sodium sulfate has an optimum between pH 4.4 and 5.0; when sodium sulfate is added this optimum shifts to pH 5.0 to 5.4 and a considerably greater activity is observed.

Figure 3 shows the pH curves with 17-hydroxyxocorticosteroid glucuronide solution. Without sodium sulfate the activities of Fraction I, II, and the starting preparation have a pH optimum between 4.0 to 4.4, between 4.4 and 4.8, and between 4.2 and 4.6, respectively. With sodium sulfate present the activities have a pH optimum between 4.4 and 4.8, between 4.6 and 5.0, and between 4.8 and 5.2, and considerably greater activity is observed. We conclude that sodium sulfate increases the activity of β-glucuronidase from bovine liver on both substrates, p-nitrophenyl- and 17-hydroxyxocorticosteroid glucuronides.

In an experiment in which we used several β-glucuronidase preparations from the same source (bovine liver), for the enzyme preparations with p-nitrophenyl-, phenolphthalein-, and 17-hydroxyxocorticosteroid glucuronides (incubation pH: 5.2, 5.2, and 5.0, respectively), we found the same effect of sodium sulfate; if the effect is expressed as the ratio of the value with sodium sulfate
than the values when it is absent. We conclude that the urinary high-molecular-weight fraction plays a major part in the inhibition of urinary \(\beta\)-glucuronidase and that such inhibition is removed by sodium sulfate.

Figure 5 shows a correlation between urinary \(\beta\)-glucuronidase inhibitory activities and total 17-hydroxycorticosteroid values in the same urines. In this experiment the hydrolysis of 17-hydroxycorticosteroid glucuronides was conducted with bovine liver \(\beta\)-glucuronidase (500 Fishman units/ml urine) in the presence of sodium sulfate (final concentration 80 g/liter, 48 °C, 18 h). When assuming that the difference between \(\beta\)-Gl, and \(\beta\)-Gl, reflects the activity of the high-molecular-weight inhibitors, we can say that the inhibitor action increases logarithmically as 17-hydroxycorticosteroid glucuronides increase in urine. This can be one of the main reasons for decrease in the efficiency of the enzymatic hydrolysis in urines containing large amounts of such glucuronides.

Effect of Sodium Sulfate on Urine Putrefaction during Incubation

We adjusted the pH of 32 urine samples obtained from hospitalized patients to pH 5.0 with diluted sulfuric acid, and then incubated without acetate buffer (37 or 48 °C, 18 h). Without sodium sulfate present, after incubation at 48 °C, eight of the 32 samples stayed at pH 5.0 and the pH of the rest had shifted to a mean pH of 6.4 (range, 5.4 to 8.0). With sodium sulfate present (final concentration, 80 g/liter) the pH of 16 samples stayed at pH 5.0, the rest shifted to a mean pH of 5.6 (range, 5.2 to 6.0). The results after incubation at 37 °C were almost the same.

Sodium sulfate thus inhibits the pH shift, presumably by inhibiting the growth of urinary microorganisms that destroy steroids, and an optimal pH for the enzyme hydrolysis is better maintained.

We conclude that sodium sulfate does inhibit urine putrefaction.

Effect of Sodium Sulfate and Temperature on \(\beta\)-Glucuronidase Activity

In the experiment concerned with the effect of sodium sulfate (final concentration, 80 g/liter) and of temperature on the enzymatic hydrolysis (500 Fishman units/ml urine) of urinary 17-hydroxycorticosteroid glucuronides:

(a) at the incubation temperature of 37 °C (18 h), the value \((n = 11)\) without sodium sulfate was 88 ± 2% (84–92) of that with sodium sulfate;

(b) in the presence of sodium sulfate the value \((n = 15)\) on incubation at 37 °C (18 h) was 92 ± 6% (78–104) of that at 48 °C;

(c) at the incubation temperature at 48 °C (18 h), the value \((n = 46)\) without sodium sulfate was 94 ± 3% (90–101) of that with sodium sulfate;

and (d) on incubating for 4.5, 12, and 18 h with sodium sulfate at 48 °C, the values for the first two were 97 ± 3% (91–102) and 99 ± 2% (96–100) that of the last.

Summarizing these results, the values with sodium sulfate present are always higher, whether the incubation is at 37 or 48 °C, than without; values on incubation at 48 °C are higher than those at 37 °C; and the value with sodium sulfate at 48 °C (18 h) is about 25% higher than that without sodium sulfate at 37 °C (18 h).

In our experiment starting with addition of 17-hydroxycorticosteroids glucuronides to 12 urine samples with 500 Fishman units of \(\beta\)-glucuronidase per milliliter of urine, the analytical recovery was 98 ± 1.8% (95–100) in the presence of sodium sulfate (48 °C, 18 h).

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References