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A Non-Competitive β-Glucuronidase Inhibitor in Rabbit Urine.* (26628)

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It has been suggested by Boyland(1) that urinary glucuronidase may be an important factor in induction of bladder cancer by certain aromatic amines. According to this concept, non-carcinogenic glucuronic acid conjugates of 2-amino-1-naphthol might be hydrolyzed by glucuronidase in the bladder to yield the carcinogenic 2-amino-1-naphthol (2).

In an investigation of the relationship of 2-naphthylamine metabolism to bladder tumor induction, urinary β-glucuronidase has been assayed in various species by the method of Boyland *et al.*(3). These determinations revealed marked variations in β-glucuronidase levels of various species. An unexpected finding was the apparent absence of this enzyme in urine obtained from several kinds of rabbits(4). Subsequent experiments showed, however, that rabbit urine did contain β-glucuronidase and that the enzyme could be precipitated from urine with ammonium sulfate. The failure to demonstrate β-glucuronidase activity in whole urine was due to the presence of inhibitory materials. Similar investigations indicated that glucuronidase inhibitors were also present in urine of man, dog, rhesus monkey, mouse and rat(4). These observations confirm and extend the findings of Abul-Fadl(5) and Lewis and Plaice(6) who demonstrated β-glucuronidase inhibitors in human urines. The present report deals with the partial characterization of the β-glucuronidase inhibitor(s) in rabbit urine.

Materials and methods. Two male and 2 female rabbits, inbred animals from a tuberculosis-resistant race,† were used for this study. They were maintained on a diet of Purina Rabbit Pellets with water *ad libitum.*

During experimental periods the animals were housed in metabolism cages equipped with stainless steel funnels; urine was collected into iced vessels, without preservative,

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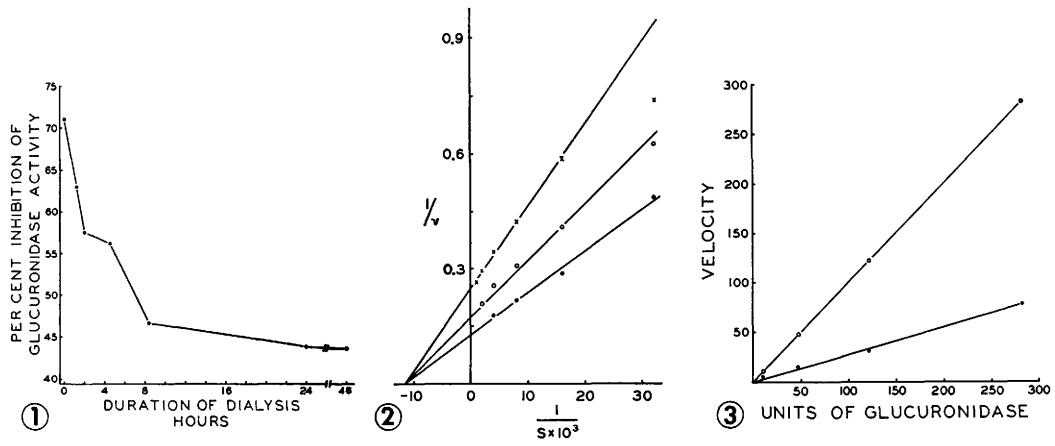

FIG. 1. Reduction in glucuronidase-inhibitor content of rabbit urine after varying periods of dialysis.

FIG. 2. Non-competitive inhibition of β-glucuronidase by rabbit urine dialysate. Reciprocal of initial velocity is plotted against reciprocal of phenolphthalein glucuronide concentration. Velocity expressed as micrograms of phenolphthalein liberated per ml per hour. Reaction mixtures contained 0.2 ml 0.2 M acetate buffer (pH 4.5), 0.2 ml enzyme solution and phenolphthalein glucuronic acid in concentrations varying from 3.125×10^{-5} to 1.0×10^{-3} M in a total vol. of 0.6 ml. Curve A (●——●), obtained in absence in inhibitor; Curve B (○——○), obtained in presence of 0.2 ml 1:8 dilution rabbit urine dialysate; Curve C (×——×), obtained in presence of 0.2 ml of 1:2 dilution rabbit urine dialysate.

FIG. 3. Relationship of reaction velocity (v) to β-glucuronidase concentration in presence and absence of urinary inhibitor. Velocity expressed as micrograms of phenolphthalein liberated per ml per hour from 0.4 mM phenolphthalein glucuronide at pH 4.5. Inhibitor concentration: ○——○, nil; ●——●, 1:3 dilution of rabbit urine dialysate.

for 24-hour periods. This chilled urine was used within a few hours after collection.

β-glucuronidase activity was determined by a slight modification of the method of Boyland *et al.*(3), using phenolphthalein glucuronic acid‡ as substrate and either *Ketodase* or Worthington β-glucuronidase as enzyme source. Unless otherwise indicated, the reaction was carried out at 37°C in 0.2 M acetate buffer, pH 4.5, with appropriate control and reagent blanks. The action of the enzyme was halted by addition of 10% sodium carbonate solution and glycine buffer, pH 10.6, and the reaction mixture clarified by centrifugation at 1600 × g for 15 minutes. The amount of phenolphthalein liberated was determined in a Klett-Summerson Colorimeter, using a No. 54 filter.

The mucoprotein fraction of urine was isolated from pooled 24-hour specimens of rabbit urine by the method of Anderson and Maclagan(7) and showed a typical diphenylamine reaction. It was dissolved in either acetate buffer, pH 4.5, or in a "synthetic urine," which contained inorganic ions and organic compounds at concentrations corresponding to those found in normal urine.

For the dialysis studies, urine samples were placed in cellulose tubing and were dialyzed at 4°C for 24 hours against running distilled water.

Results and discussion. The anti-glucuronidase activity of rabbit urine can be reduced but not eliminated by dialysis against cold running distilled water (Fig. 1). The loss of diffusible inhibitory material proceeds fairly rapidly during the first 8 hours of dialysis, then slows down markedly. The non-dialyzable fraction remaining after 48 hours of dialysis represents 60% of the total inhibitor present in urine.

Subjecting rabbit urine which had been dialyzed for 24 hours to autoclaving for 15 min at 15 lb pressure had no effect on the activity of the non-dialyzable inhibitor, indicating that it was heat-stable, but the ash from urine dialysates contained no glucuronidase inhibitors. This inhibitor therefore appears to bear a resemblance to the non-dialyzable, heat-stable, protein-like inhibitor(s) found

‡ Sigma Chemical Co.

TABLE I. Effect of Hydrogen Ion Concentration on Activity of Urinary Glucuronidase Inhibitor(s).

pH	% inhibition	pH	% inhibition
3.0	34.4	5.5	51.4
3.5	42.2	6.0	42.2
4.0	44.2	6.5	6.7
4.5	45.9	7.0	.0
5.0	47.2		

in plasma by Fishman(8), and to an inhibitor(s) extracted from urine by Abul-Fadl (5). However, isolation of the mucoprotein fraction from rabbit urine by benzoic acid precipitation(5) yielded a product which was devoid of anti-glucuronidase activity.

Assay of the non-dialyzable, heat-stable inhibitor in rabbit urine at various pH levels indicates that it is active over a pH range from as low as 3.0 to approximately 6.0, with a maximum at pH 5.5 (Table I). Furthermore, the results obtained in kinetic studies agree closely with those expected for pure non-competitive inhibition(9). This was concluded from the following experiments.

The reaction velocity of β-glucuronidase was determined without inhibitor and in the presence of rabbit urine dialysate at 2 concentrations (1:2 and 1:8). When the reciprocals of the reaction velocities (1/v) are plotted against the reciprocals of substrate concentration (1/S), curve A (control) intercepts the 1/v axis at 0.13, corresponding to the liberation of 3.9 γ phenolphthalein ml hr. The corresponding intercepts for curve B (1:8 urine dialysate) and curve C (1:2 urine dialysate) were 0.18 and 0.25, equivalent to maximum velocities of 2.8 and 2.0 γ/ml/hr. The substrate concentration giving half maximum velocity (Km), which was unchanged by the presence of inhibitor, was 8.7×10^{-5} M.

To exclude the possibility of "pseudo-irreversible" inhibition, the test of Ackerman and Potter(10) was applied (Fig. 3). The resulting curves, representing velocity of hydrolysis as a function of glucuronidase concentration, in presence and absence of a constant amount of inhibitor, were indicative of reversible inhibition.

These investigations indicate that the failure of rabbit urine to hydrolyze glucuronic acid conjugates is due not to absence of glucuronidase, but to the presence of a glucuronidase inhibitor(s) which functions in a non-competitive fashion. They suggest that the resistance of rabbits to bladder carcinogens such as 2-naphthylamine may be attributable to inhibition of urinary β-glucuronidase, and the consequent inadequate hydrolysis of 2-amino-1-naphthyl glucuronide to the carcinogen, 2-amino-1-naphthol.

Summary. A heat-stable, non-dialyzable inhibitor of β-glucuronidase is present in rabbit urine. It is not associated with the mucoprotein fraction of urine. Kinetic studies indicate the substance(s) functions as a non-competitive inhibitor, which combines reversibly with the enzyme.

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