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Effect of Activators on the Formation of Diethylstilbestrol Diglucuronide by Hamster Liver Homogenates *

The procedures described until now (1, 12) for the extraction of DESGA ** from the urine of rabbits fed DES are tedious and slow when applied to large volumes of urine and in order to avoid these inconveniences we have utilized a new procedure by taking into account the property of glucuronides for the formation of insoluble lead salts (9).

Urine was acidified with concentrated HNO_3 until *p*H 4; 50 g of normal lead acetate were added per liter of acidified urine and stored 2 hours at 5° C. The DESGA formed lead salt was separated by centrifugation and the urine discarded; the sediment was washed twice with cooled 5 % normal lead acetate. The amount of DESGA lost in these operations was about 52 mg/l. Sediments were suspended in water and acidified with 6N H_2SO_4 up to *p*H 1.7-2 in order to free DESGA from its lead salt. The pasty mass was homogenized with ether by means of an electric whisk. The ethereal layer was separated by centrifugation and the extraction of the pasty mass was repeated several times. The pooled ethereal layers were concentrated and $HNaCO_3$ was added until all DESGA was neutralized. The precipitate of the sodium salt of DESGA was separated by centrifugation. From this point a slight modification of the procedure described by DODGSON *et al.* (1) was followed. The final extracts obtained with the technique utilized are much purer than the ones obtained with the techniques described before, which makes possible a greater yield of DESGA in the crystalli-

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** The abbreviations used are: DES, diethylstilbestrol; DESGA, diethylstilbestrol monoglucuronide; UDP, uridine diphosphate; UDPGA, uridine diphosphate glucuronic acid; UDPAG, uridine diphosphate N-acetylglucosamine; EDTA, ethylene diamine tetraacetic acid.

zation process. The product obtained had m.p. 176° and was chromatographically pure in the systems described previously (6).

Once DESGA was obtained we attempted to ascertain whether it could act as a substrate for glucuronyltransferase (UDP-glucuronate glucuronyltransferase, EC 2.4.1.17). The formation of diglucuronides in the case of aglycons with more than one functional group available for conjugation seems to be rare. The *in vitro* formation of small amounts of estrogen diglucuronides has been reported (10). The best known and most widespread diglucuronide is probably that of bilirrubin in bile (7). FISCHER *et al.* (3) found, apart from the monoglucuronide, a small amount of a compound they thought to be the diglucuronide in the bile of rats injected with DES. The fact that rat liver formed the diglucuronide of DES led us to suppose that hamster liver could also form it, since this animal is much more efficient than rat in the production of DES monoglucuronide (5).

For the study of DESGA consumption by hamster liver homogenates a standard curve was prepared with DESGA concentrations up to 130 μg/ml in a similar way to that described for DES in a previous work (6). Hamster liver homogenates were prepared as described previously (6). The composition of the incubation mixture was: tris, 32.8 mM; $MgCl_2$, 9.8 mM; saccharolactone, 3 mM; UDPGA, 0.42 mM; DESGA, 0.27 mM; propylene glycol, 2.3 % v/v; 15 % homogenate, 0.5 ml (final volume, 2.5 ml; *p*H, 7.3). DESGA was added to the incubation mixture dissolved in 41 % propylene glycol. The concentration of the activators was: Triton X-100, 0.05% v/v; ATP, 1 mM; UDPAG, 0.5 mM; EDTA, 10 mM; preincubation was carried out during 2.5 hours at 37°. The mixture was incubated with shaking at 37° for 90 min. The reaction was stopped in a boiling water bath during 10 minutes; 1.5 ml of ethanol were added to the tubes when cooled and the content filtered; 0.4 ml of H_2O and the same reagents as in the standard curve were added to an aliquot of 0.6 ml from the filtrate. The amount of DESGA utilized was calculated as the difference between the blanks incubated without substrate and with DESGA added just before protein precipitation, and the samples incubated with DESGA.

Hamster liver homogenates did not consume DESGA either in the absence or in the presence of activators (Triton X-100, ATP + UDPAG, Triton + ATP + UDPAG, EDTA and preincubation). This contrasts with the strong effect activator of the compounds tested when assayed on the reaction of DES glucuronidation (4). The formation of DES diglucuronide has not been detected either in everted sacs of rat intestine (2) or in liver and kidney homogenates of rabbits, hens, guinea pigs and cats when incubated with DES and UDPGA (11).

The kinetic analyses performed by HANNINEN and MARNIEMI (8) indicate that the inhibition of the glucuronide synthesis by glucuronides is due to a competitive mechanism between the glucuronide and the UDPGA. The competition between DESGA and UDPGA to join the UDPGA binding site of glucuronyltransferase may be an explanation of the fact that DESGA, in spite of possessing one free phenolic group, does not form the diglucuronide.

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