Glucuronosyl Transferase Activity in Livers of Rat, Dog, and Sheep.* (29320)

LEGRANDE C. ELLIS† AND W. STANLEY NEWCOMER
Department of Physiology and Pharmacology, Oklahoma State University, Stillwater

Under normal conditions, ruminant animals are presented with large amounts of ionone derivatives of dietary origin(1,2,3). These compounds, although lacking any known physiologic function, are conjugated with both glucuronic acid(4) and sulfuric acid(2) and are excreted in a manner similar to that of steroids. The purposes of this investigation were to measure and compare the intrinsic capacity of the glucuronyl transferase system to conjugate various substrates in hepatic tissue from a ruminant animal with that from non-ruminant animals.

Materials and methods. Ten western type, female sheep were maintained on pasture and a prairie hay supplement with water given *ad libitum*. Five mature, female rats (Holtzman strain) weighing 300-360 g were maintained on laboratory chow and water *ad libitum*. Five mongrel dogs were maintained on Purina dog chow and water *ad libitum*.

Livers, obtained from rats after decapitation and from dogs and sheep after electrocution, were immediately chilled, weighed, and minced with scissors. All tissues were homogenized in ice-cold KCl solutions with Potter-Elvehjem homogenizers. A 0.1 M KCl solution was used for microsomal preparations(5) while isotonic KCl was used for homogenate preparations. A 30% (W/V) homogenate was made for all studies. Microsomal preparations were prepared by centrifuging the appropriate homogenates in a refrigerated centrifuge (Servall) at 10,800 × *g* for 10 minutes (2-3°). The resulting supernatant solution was decanted and centrifuged for one hour in a preparative ultracentrifuge (Spinco model L) at 105,000 × *g*. The supernatant fluid was removed and the resulting microsomal pellet was resuspended in 0.1 M KCl with the aid of a small homogenizer. Each ml of resuspended microsomes represented 2½ g of original tissue.

Homogenates were incubated in Warburg flasks containing: 1.0 ml of homogenate, 25 mg of glucose, 0.5 ml of buffer(5), 0.5 ml of UDPGA‡ (0.086 micromoles—Sigma Chemical Co.), and 0.5 ml aqueous solution containing 0.140 micromoles of steroids (stock solutions of steroids were dissolved in several drops of methanol and diluted with water to 50 ml). Non-incubated, control samples were prepared as described above, however UDPGA in these tubes was replaced with 0.5 ml glass-distilled water. These test tubes were kept in an ice-bath while the Warburg flasks were incubated for 2½ hours at 37° under atmospheric oxygen. Incubations of microsomal preparations were carried out in 15 × 125 mm test tubes under constant shaking at 37° for one hour. The steroids and dyes (dissolved in methanol) were added to the test tubes and blown to dryness with nitrogen just prior to adding the buffer and other substrates. Two tubes were used for each assay with one tube being incubated and the other tube remaining in an ice-bath to serve as a non-incubated control. The tube which was incubated contained 0.1 ml UDPGA (0.4 micromoles), 0.55 ml Tris buffer(6), and one ml of the microsomal preparation. Similarly, the non-incubated tube contained the solutions mentioned above except that the UDPGA was replaced with 1.0 ml glass-distilled water.

Following incubation, the contents of the Warburg flasks were quantitatively transferred to test tubes and the test tubes of both

* Published with approval of Director of Oklahoma Agri. Exp. Station.

† Present address: Dept. of Anatomy, Univ. of Utah College of Med., Salt Lake City.

‡ The following abbreviations are used in this paper: UDPGA, uridine diphosphoglucuronic acid; Tris, tris (hydroxymethyl) aminomethane; H_4E, 5β-pregnane-3α, 17α, 21-triol-11,20-dione; H_4F, 5β-pregnane-3α, 11B, 17α, 21-tetraol-20-one; p-NP, p-nitrophenol; phen., phenolphthalein; UDP-n-acetyl-glucosamine, uridine diphospho-N-acetyl-glucosamine; and UDPG, uridine diphosphoglucose.

the incubated and non-incubated series for homogenate and microsomal studies were placed in a boiling water bath for 2 minutes. All tubes containing steroids were extracted 3 times with 2½ volumes of chloroform (redistilled from anhydrous K_2CO_3); the pooled extracts were partitioned against 0.1 N NaOH and 0.1 N HCl. The resulting chloroform extracts were evaporated to dryness and the steroids were separated from interfering materials by column chromatography. Each sample (in 5 ml chloroform) was added to a Florisil (Floridin Co.) column(7,8). The column was developed with 25 ml of chloroform, 10 ml of 3% (V/V) methanol in chloroform, 60 ml of 3% (V/V) methanol in chloroform, and finally 20 ml of 40% (V/V) methanol in chloroform. With the aid of known steroids, it was shown that the latter 2 fractions contained the steroids H_4E and H_4F, respectively. The appropriate fractions of the eluant were blown to dryness and analyzed for steroid content by the Robertson-Mixner modification of the Porter-Silber method(9). The samples were read in a spectrophotometer (Beckman model DU) with micro cells (Pyrocell—1 cm light path).

Phenolphthalein and p-nitrophenol determinations were accomplished after precipitation of the proteins with 3 ml of 95% ethanol. Following removal of the precipitate by centrifugation, one ml aliquots were taken from each tube for assays which were run in duplicate. Phenolphthalein was assayed according to the method for β-glucuronidase studies (Sigma Chemical Co., revised bulletin—105). The method of Isselbacher and McCarthy(6), was used for p-nitrophenol studies. Samples from both assays were read in a spectrophotometer (Coleman, Jr.). Micromole quantities of substrates disappearing after incubation were obtained by differences between non-incubated and incubated tubes.

Following extraction of the media containing the steroids with chloroform, the resulting aqueous residue containing the conjugated steroids was incubated with β-glucuronidase (Ketodase)(10) and the free steroids were extracted with chloroform and separated from interfering materials as mentioned earlier. The statistical significance of substrates disappearing after incubation and recovery of steroids after hydrolysis with β-glucuronidase was ascertained with a "t"-test(11). Electrophoretic separation of steroid conjugates was accomplished by means of conventional procedures(12).

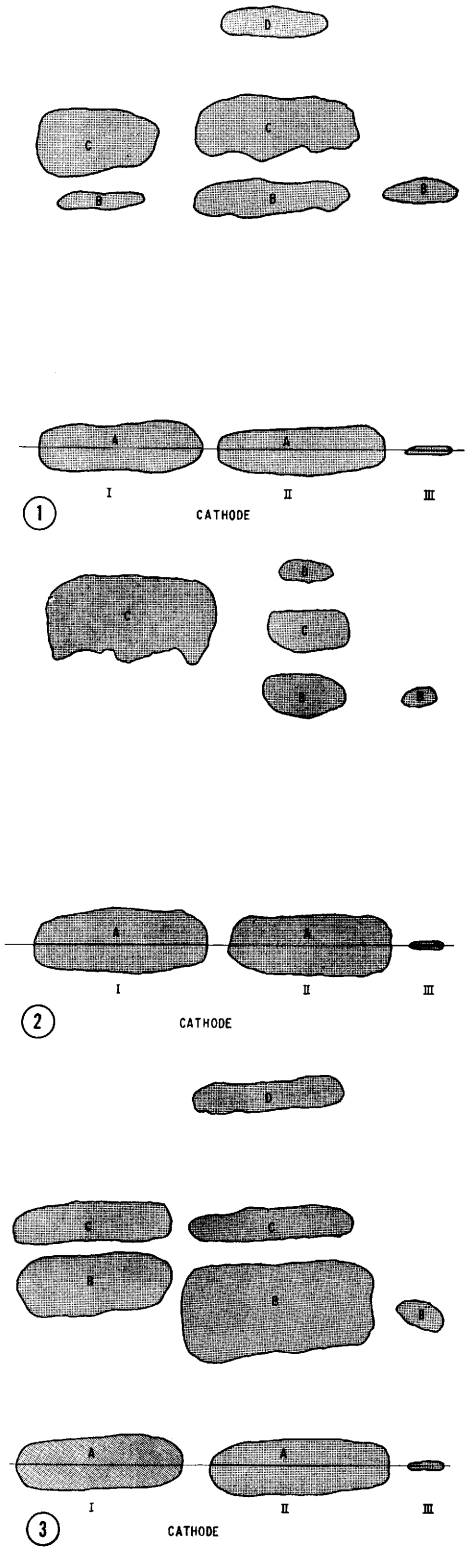
TABLE I. Glucuronide Conjugation of H_4E by Homogenates of Liver Tissue from Sheep, Dog, and Rat.

Animal	No. of observations	Substrate disappearing on incubation	Substrate recovered as glucuronide
		(μmoles)*	(μmoles)*
Sheep	10	.101 ± .025†	.047 ± .017†
Dog	5	.038 ± .012†	(−).005 ± .003†
Rat	5	.073 ± .022†	.009 ± .005†

* Mean ± standard deviation.
† Statistical probability $<.05$ as compared with non-incubated controls.

Results. Sheep liver homogenates demonstrated a greater capacity to conjugate H_4E than did liver preparations from dogs or rats (Table I). Dog liver preparations failed to demonstrate the ability to conjugate steroids on the basis of the ability to recover H_4E from the incubation media following hydrolysis with β-glucuronidase. In fact, a net hydrolysis of endogenous steroid conjugates was observed. When sheep liver microsomal preparations were incubated with H_4E, H_4F, phenolphthalein, and p-nitrophenol (Table II), the amounts of these substrates disappearing and reappearing after hydrolysis with β-glucuronidase indicate that sheep liver preparations have a greater ability to conjugate various substrates than do those from either the rat or dog. However, dog liver microsomal preparations were able to conjugate both p-nitrophenol and phenolphthalein at a rate greater than similar preparations from rats. In addition, electrophoretic separation of steroids (Fig. 2) indicated that the dog can conjugate H_4E in small quantities. With this exception, the data from the electrophoretic separation of steroids (Figs. 1, 2, 3) correlate well with the Porter-Silber assays (Table I).

When sheep liver homogenates were incubated with glucose or galactose as a potential UDPGA regeneration system, and phenolphthalein and p-nitrophenol were used as test substances, 0.040 micromoles of phenol-



phthalein were conjugated in contrast to 0.020 micromoles which were conjugated when galactose was used. Similarly, 0.041 micromoles of p-nitrophenol were conjugated when glucose was used as a potential UDPGA generating system in contrast to 0.034 micromoles which were conjugated when galactose was used as a precursor for UDPGA.

Discussion. These data indicate that sheep liver has a greater ability to conjugate H_4E, p-nitrophenol, and phenolphthalein than do similar preparations from dogs or rats. Perhaps the presence of large quantities of exogenous materials such as ionone derivatives of dietary origin may affect the ability of the liver parenchymal cells to conjugate various substrates such as steroids (H_4E and H_4F) or phenols (p-nitrophenol and phenolphthalein). In this respect, it has been proposed that the reticuloendothelial cells of liver are responsible for Δ^4-reductase activity while the liver parenchymal cells are responsible for glucuronosyl transferase activity(13).

There are, however, at least 2 parameters to consider when evaluating transferase activity of a given tissue: rate of synthesis of UDPGA from UDP and glucose or galactose and amount of transferase activity present in the tissue. To evaluate the first parameter, homogenates of the tissues were used and UDPGA was added in catalytic amounts (0.086 micromoles) so that the amount of UDPGA present would be rate limiting. In this manner, glucose or galactose could serve as a potential regenerating system for UDPGA. That these two compounds did serve as precursors in the regeneration of UDPGA is readily ascertained by the fact

FIG. 1. Electrophoretic pattern of steroid extracts from rat liver homogenate preparations (incubated and non-incubated) showing: A, free steroids; B, glucuronide conjugated steroids; C, steroids having a mobility similar to sulfate conjugated steroids; and D, unknown substance having a mobility similar to glucuronic acid; I, non-incubated; II, incubated; III, reference standard.

FIG. 2. Electrophoretic pattern of steroid extracts from dog liver homogenate preparations (incubated and non-incubated) with groups of steroids as indicated in Fig. 1.

FIG. 3. Electrophoretic pattern of steroid extracts from sheep liver homogenate preparations (incubated and non-incubated) with groups of steroids as indicated in Fig. 1.

TABLE II. Glucuronide Conjugation of Various Substrates by Microsomal Preparations of Liver Tissue from Various Animals.

Animal	Substrate	No. of observations	Substrate disappearing on incubation	Substrate recovered as glucuronide
			(μmoles)*	(μmoles)*
Sheep	H_4E	5	.178 ± .006†	.092 ± .003†
"	H_4F	5	.126 ± .023†	.104 ± .058†
"	Phen	5	.184 ± .049†	
"	p-NP	5	.140 ± .028†	
Dog	H_4E	5	.040 ± .007†	.0002 ± .0001‡
"	Phen	5	.072 ± .015†	
"	p-NP	5	.063 ± .017†	
Rat	H_4E	5	.025 ± .015†	.006 ± .003†
"	Phen	5	.028 ± .015†	
"	p-NP	5	.020 ± .007†	

* Mean ± standard deviation.
† Statistical probability $<.05$ as compared with non-incubated controls.
‡ Statistical probability $<.10$ as compared with non-incubated controls.

that addition of glucose to sheep liver preparation resulted in conjugation of larger amounts of phenolphthalein and p-nitrophenol than when galactose was added to the incubation mixture. In this respect, galactose has been reported to be a better precursor for glucuronic acid synthesis than glucose(14) in rats. Furthermore, two mechanisms have been proposed whereby galactose can be converted to UDPG(15). However, it was proposed that the phosphorylation of galactose is the rate-limiting step in conversion of galactose to UDPG by these two pathways. From these data (Table I) it is inferred that sheep liver has a greater capacity to synthesize UDPGA from glucose than do similar preparations from dogs or rats.

The second parameter was evaluated through the use of microsomal preparations since glucuronosyl transferase is found in this particulate fraction(16-18) and the UDPGA-pyrophosphorylase and UDPGA-dehydrogenase enzymes are found in the particulate-free fraction(19,20). Therefore larger amounts of UDPGA (0.4 micromoles) were added to the microsomal preparations so that the limiting factor in the synthesis of glucuronide-conjugates would be the amount of transferase enzyme present in the microsomal preparations. From these data (Table II) it appears that sheep liver contains more glucoronosyl transferase activity than do similar preparations from dogs or rats. In this respect, the liver of the dog appears to be a poor conjugator of steroids. W. Stevens (personal communication) indicates that dog liver minces do conjugate small amounts of steroids. Furthermore, this activity is greatly enhanced by addition of UDPGA, but was not enhanced by nucleotide activation with UDP-N-acetylglucosamine as has been reported for some species(21). Another recent report(22) indicates that dog livers perfused with certain steroids readily form sulfate conjugates with some H_4E-glucuronide being formed under these conditions. Although perfused dog kidney can conjugate etiocholanolone and androsterone with glucuronic acid(23), other indirect evidence(24,25) indicates that the liver of dogs is important in removal of C^{14}-labeled steroids from the blood stream.

It is possible that other mechanisms could be responsible for the species difference noted in this investigation for the conjugation of various substrates with glucuronic acid. However, it appears likely that the large quantities of ionone derivatives of dietary origin which these animals consume could be responsible for this increased activity. In this respect, it has been shown(26,27) that administration of glucan, a reticuloendothelial cell stimulating agent, can increase the ability of the liver to reduce the Δ^4-3-keto grouping of ring A of some corticosteroids. Other studies(28) indicate that the Δ^4-reductase

activity of sheep livers is actually lower than that observed for rats.

Summary. Rat, sheep, and dog liver homogenates and microsomal preparations were assayed for glucuronosyl transferase activity and ability to regenerate UDPGA from glucose. Sheep liver displayed a greater capacity to conjugate H_4E, p-nitrophenol, and phenolphthalein than did similar preparations from dogs and rats. This enhanced ability was attributed to an increase of glucuronosyl transferase activity and the ability to regenerate UDPGA from glucose. In addition, glucose was observed to be a better precursor for regeneration of UDPGA than galactose with sheep homogenate preparations. Dog liver was found to conjugate H_4E poorly, but was able to conjugate p-nitrophenol and phenolphthalein.

The authors would like to thank Dr. John J. Schneider for his generous gift of H_4E-glucuronide which was used as a reference standard in the electrophoretic studies.

1. Holtz, A. H., *Acta Endocrinol.*, 1957, v26, 75.
2. Ungar, F., Rosenfeld, G., Dorfman, R. I., *Am. J. Vet. Res.*, 1961, v22, 213.
3. Ellis, L. C., *Thesis,* Oklahoma State Univ., Stillwater, 1961.
4. Mixner, J. P., Saunders, H. L., Jr., Johnson, J. E., *J. Dairy Sci.*, 1957, v40, 67.
5. Potter, V. R., Pardee, A. B., Lyle, G. G., *J. Biol. Chem.*, 1948, v176, 1075.
6. Isselbacher, K. J., McCarthy, E. A., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 819.
7. Nelson, D. H., Samuels, L. T., *J. Clin. Endocrinol. Metab.*, 1952, v12, 519.
8. Gardner, L. I., *ibid.*, 1953, v13, 941.
9. Robertson, W. G., Mixner, J. P., *J. Dairy Sci.*, 1956, v39, 589.
10. Glenn, E. M., Nelson, D. H., *J. Clin. Endocrinol. Metab.*, 1953, v13, 911.
11. Snedecor, G. W., *Statistical Methods,* Iowa State College Press, Ames, 1949.
12. Cohn, G. L., Bondy, P. K., *J. Biol. Chem.*, 1959, v234, 31.
13. Berliner, D. L., Dougherty, T. F., *Ann. N. Y. Acad. Sci.*, 1960, v88, 14.
14. Touster, O., *Fed. Proc.*, 1960, v19, 977.
15. Segal, S., Roth, H., Bertolo, D., *Science,* 1963, v142, 1311.
16. Dutton, G. J., *Biochem. J.*, 1956, v64, 693.
17. Dutton, G. J., Storey, I. D. E., *ibid.*, 1953, v53, XXXVII.
18. Isselbacher, K. J., Axelrod, J., *J. Am. Chem. Soc.*, 1955, v77, 1070.
19. Strominger, J. L., Kalcar, H. M., Axelrod, J., Maxwell, E. S., *ibid.*, 1954, v76, 6411.
20. Storey, I. D. E., Dutton, G. J., *Biochem. J.*, 1955, v59, 279.
21. Pogell, B. M., Leloir, L. F., *J. Biol. Chem.*, 1961, v236, 293.
22. Tamm, J., Voigt, K. D., Volkwein, U., Berle, P., *Steroids,* 1963, v2, 279.
23. Cohn, G. L., Hume, M., *J. Clin. Invest.*, 1960, v39, 1584.
24. Kuipers, F., Ely, R. S., Kelly, V. C., *Endocrinol.*, 1958, v62, 64.
25. Willoughby, H. W., Chen, C., Freeman, S., *ibid.*, 1959, v65, 539.
26. Berliner, D. L., Nabors, C. J., Jr., Dougherty, T. F., *J. Reticuloendothelial Soc.*, 1964, v1, 1.
27. Nabors, C. J., Jr., Gallegos, A. J., Berliner, D. L., 45th Meeting of Endocrine Soc., 1963.
28. Ellis, L. C., Newcomer, W. S., PROC. SOC. EXP. BIOL. AND MED., 1964, v116, 617.

Received March 2, 1964. P.S.E.B.M., 1964, v116.

Δ^4-Reductase and Sulfokinase Activities of Rat and Sheep Liver Preparations.* (29321)

LEGRANDE C. ELLIS† AND W. STANLEY NEWCOMER
Department of Physiology and Pharmacology, Oklahoma State University, Stillwater

It has been pointed out by other workers (1,2,3) that ruminant animals are normally presented with large amounts of ionone derivatives of dietary origin. These compounds react with many of the colorimetric reagents in tests for steroids(2,4,5) and are known to

* Published with approval of Director of Oklahoma Agri. Exp. Station.

† Present address: Dept. of Anatomy, Univ. of Utah College of Med., Salt Lake City.