PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 189-193 (1978)

Glutathione-S-Transferase Activity in the Rabbit Nephron: Segmental Localization in Isolated Tubules and Formation of Thiol Adducts of Ethacrynic Acid (40018)

L. G. FINE,* E. J. GOLDSTEIN,† W. TRIZNA,* L. ROZMARYN,* AND I. M. ARIAS†

Division of Nephrology, University of Miami School of Medicine, Miami, Florida 33152, †Liver Research Center, Albert Einstein College of Medicine, The Bronx, New York 10461

Ligandin is a basic protein isolated from the supernate of rat and human kidney and liver homogenates (1, 2). In the rat and man it is the major glutathione (GSH)-S-transferase (3). Conjugation with GSH is the first step in mercapturate formation which is the major nonoxidative detoxification system for many drugs and chemicals including ethacrynic acid (4). Administration of ethacrynic acid results in formation of glutathione (EA-GSH) and cysteine (EA-cyst) adducts of the diuretic (5). The latter has a significantly greater diuretic effect on the isolated thick ascending limb of Henle's loop than does ethacrynic acid (6). The present studies were designed to localize GSH transferase activity in the nephron and to determine its role in formation of thiolated adducts.

Methods. Isolation of nephron segments. Studies were performed on rabbits since the nephron segments of this species can be identified and isolated by microdissection (7, 8). Normal female white New Zealand rabbits, approximately 2 kg in weight, were maintained on a diet of standard rabbit chow. The animals were killed by cervical dislocation and the left kidney was immediately removed. A 1- to 2-mm-thick transverse section and a 1- to 2-mm tangential section of superficial cortex were removed and transferred to a dish of chilled McCoy's 5A cell culture medium without phenol red (Grand Island Biological Co., N.Y.), which also served as the dissecting medium. The medium was bubbled with 95% O_2–5% CO_2. Individual tubular segments were separated by microdissection using a dissecting microscope with a calibrated eyepiece reticle. Proximal convoluted tubules were dissected from the superficial cortical section and the other tubular segments from the transverse section. Proximal convoluted tubules, proximal straight tubules, combined thin descending and ascending limbs of Henle, combined cortical and medullary thick ascending limbs, and combined cortical and medullary collecting tubules were separately isolated. Accuracy of identification was confirmed by measuring the characteristic transtubular potential difference in representative segments using the *in vitro* perfusion technique (7, 8). An approximate total length of 20–40 mm of each segment was pooled in separate test tubes containing approximately 0.2 ml of chilled dissecting medium. Tubular segments were washed twice with 20 ml of medium and centrifuged to remove enzyme liberated during dissection. Aliquots (0.2 ml) of medium were similarly treated and assayed for enzyme activity and did not have GSH transferase activity. Aliquots (0.4 ml) of medium containing the tubular segments were rapidly frozen, thawed, and sonicated for 15 sec in a Sonifier cell disruptor (Heat Systems-Ultrasonic, Inc., Plainview, N.Y.). Total protein was determined using the method of Lowry *et al.* (9). GSH transferase activity was determined on 0.1 ml of supernate representing a total length of 5–10 mm of tubule.

GSH transferase assay. Glutathione transferase activity was determined by monitoring formation of enzyme-catalyzed coupling between glutathione and 1-chloro-2,4-dinitrobenzene (E 9600 at 340 nm) (4).

Aliquots (100 μl) of tubule supernate in bicarbonate buffer (pH 7.4) were added to a 1-cm-path spectrophotometer cell containing 3 ml of a solution of 2.5 mM glutathione and 1.33 mM 1-chloro-2,4-dinitrobenzene in 0.1 M phosphate buffer at pH 7.4. The blank cell contained only reagents. Changes in absorption were followed at 25° in a Pye Unicam 1800 recording spectrophotometer.

0037-9727/78/1572-0189$01.00/0

Units of enzyme activity were expressed as micromoles of coupling product formed per minute per microgram of protein.

Formation and liberation of ethacrynic acid adducts by intact tubules. Slices (1 mm) of superficial renal cortex and papillary tip were transferred to a dish of chilled ultrafiltrate of normal rabbit serum prepared by ultrafiltering serum through a Diaflo PM membrane (Amicon Corp., Lexington, Mass.) which effectively excludes molecules of greater than 10,000 MW. The ultrafiltrate was used as the dissecting and incubation medium. (Binding of ethacrynic acid to serum proteins was greater than 99% at 37°.) Teased tubule preparations were prepared from cortical and papillary slices by gentle separation of tubule fragments with fine forceps under a dissecting microscope. The superficial cortical slice was composed almost entirely of proximal tubules, whereas the papilla contained only loops of Henle and collecting tubules.

Two samples of each preparation (10%, w/v) were transferred to individual wells of a microtiter plate (Falcon Plastics) and incubated in ultrafiltrate containing 10^{-4} *M* [^{14}C]oxylacetic-2-ethacrynic acid[1] (sp act, 16.4 μCi/mg) for 30 min at 37° in a humidified atmosphere of 95% O_2–5% CO_2. The protein content of each well was determined by the method of Lowry *et al.* (9). The concentration of EA adducts in the incubation medium did not increase between 15 and 30 min of incubation. Two samples of the isotope-containing ultrafiltrate without tissue were also incubated simultaneously. The incubation medium was collected into capillary tubes and 10 μl was placed on precoated silica gel F-254 thin-layer chromatography plates (Brinkman Instruments, Westbury, N.Y.). Each sample was superimposed upon a spot containing 10% ethacrynic acid and 10% ethacrynic acid–glutathione adduct (prepared in crystalline form and provided by Dr. D. Koechel, Department of Pharmacology and Therapeutics, Medical College of Ohio, Toledo, Ohio). The plates were developed in benzene:methanol:acetic acid (45:8:4). Ethacrynic acid and ethacrynic acid–glutathione spots were localized under uv light (253.7 nm), and individual areas were scraped and transferred to counting vials containing 10 ml of Aquasol liquid scintillation fluid (New England Nuclear) for radioactive counting.

The area between the ethacrynic acid and ethacrynic acid–glutathione spots contained ethacrynic acid adducts of intermediate molecular weight. This region was removed and counted separately. Two 10-μl samples of the incubation medium from each well were pipetted directly into counting vials. Samples were counted in a Packard–Tricarb liquid scintillation spectrophotometer with correction for quenching. Total recovery of isotope from the plate was expressed as a percentage of counts obtained by direct counting of an equal volume of the same sample. Absolute values were obtained from the thin-layer plates for this recovery factor which exceeded 80% in all experiments. Ninety-six percent of [^{14}C]ethacrynic acid radioactivity was recovered from the ethacrynic acid spot, 2.5% from the ethacrynic acid–glutathione spot, and 1.5% from the intermediate zone.

In order to determine the proportion of ethacrynic acid–cysteine adduct (EA–Cys) in the "unidentified adducts" obtained from the thin-layer plates, three additional experiments were performed on proximal tubule suspensions in which separation of the ethacrynic adducts was achieved by paper chromatography as described by Duggan and Noll (10). An EA–Cys marker was prepared by dissolving equimolar amounts of ethacrynic acid and L-cysteine in water. Because free cysteine was present, the samples were applied to the paper adjacent to the EA, EA–GSH, and EA–Cys markers to prevent formation of EA–Cys by its combination with free ethacrynic acid in the sample. The area between the EA and the EA–GSH zones was removed after development and divided into an EA–Cys section and a remaining section containing other unidentified adducts. Radioactivity was counted using Toluene Puresolve liquid scintillation cocktail (Packard Instruments Co., Downers Grove, Ill.).

Tissue content of ethacrynic acid adducts. Following incubation, proximal tubules

[1] Supplied by Dr. Frank Wolf, Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey.

were blotted dry, rapidly washed in incubation medium, transferred to small tubes containing 100 μl of benzene:methanol: acetic acid (45:8:4), disrupted by sonication, and extracted for 1 hr. The extracts were separated on thin-layer plates as described above.

Results. Localization of glutathione transferase activity. Enzyme activity was confined to the proximal convoluted and proximal straight tubules and was not detectable in the thin descending and ascending loops, thick ascending limbs, or cortical and medullary collecting tubules.

Activity (μmoles of conjugate/μg of protein/min) was 0.23 ± 0.04 in the convoluted portion and 0.25 ± 0.09 in the straight portion of the proximal tubules.[2] Duplicate measurements on the same sample gave identical results.

Formation of ethacrynic acid adducts by intact tubules. a) Spontaneous formation in incubation medium. Figure 1 illustrates the fact that following incubation of an ultrafiltrate of rabbit serum with [^{14}C]ethacrynic acid (10^{-4} *M*) in the absence of renal tissue, radioactivity recoverable as ethacrynic acid was reduced to 7.4 ± 1.1 × 10^{-5} *M* concomitant with the appearance of EA- GSH (0.5 ± 0.2 × 10^{-5} *M*) and other EA adducts (0.8 ± 0.1 × 10^{-5} *M*).

(*b*) *Formation of adducts by renal tubules.* In five experiments, both proximal tubules and Henle's loops plus collecting tubules were removed from the same kidney. The mean protein content (±SE) per incubation well of proximal tubules was 291 ± 22 μg, and that of Henle's loops plus collecting tubules was 262 ± 36 μg.

In both groups of tubules the concentration of ethacrynic acid in the incubation medium fell significantly and the levels of EA-GSH increased. The increment obtained with proximal tubules was significantly greater than that with papillary segments ($P < 0.05$). Since GSH transferase activity was not detected in the latter nephron segments, it is assumed that this represents noncatalytically formed EA-GSH which is liberated into the incubation medium. No additional ethacrynic acid adducts were formed by the loops and collecting tubules, whereas there was a significant increase in the medium concentration of other "unidentified adducts" following incubation of proximal tubules (Fig. 1). The difference between the original medium concentration of isotope and total isotope recovery following incubation represents the amount removed by nonspecific tissue binding.

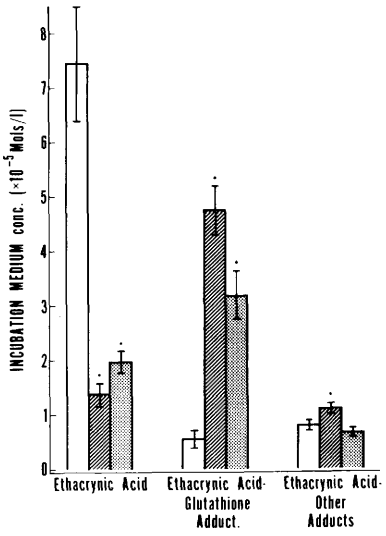

FIG. 1. Medium concentrations of ethacrynic acid, ethacrynic acid-glutathione, and other ethacrynic acid adducts following incubation of rabbit serum ultrafiltrate with [^{14}C]ethacrynic acid in the absence and presence of renal tubules. Open bars = no renal tissue; cross-hatched bars = proximal tubules; stippled bars = Henle's loops and collecting tubules. (*) $P < 0.05$ vs medium with no tissue.

Paper chromatography of the unidentified adducts (as defined by thin-layer chromatography) formed by proximal tubules revealed that 61.3 ± 9.2% of the radioactivity was recoverable as the ethacrynic acid-cysteine adduct.

(*c*) *Tissue content of ethacrynic acid and its adducts.* Analysis of the relative distribution of adducts within the proximal tubular cells revealed 15.0 ± 4.6% as ethacrynic acid, 21.3 ± 4.1% as unidentified adducts, and 63.8 ± 3.5% as ethacrynic acid-glutathione.

Discussion. Cellular localization of enzyme activity in different parts of the neph-

[2] Results are the means ± SE of enzyme assays on five animals.

ron has hitherto been based on histochemical techniques using freeze-dried sections of tissue (11). More recently a method of enzyme localization has been utilized in which isolated viable segments of the rabbit nephron are removed and assayed for enzyme activity *in vitro*. Imbert and co-workers (12) and ourselves (13) have measured adenylate cyclase activity in segments of the rabbit nephron. The present study represents an additional application of this technique. The glutathione transferase assay was sufficiently sensitive to detect activity in proximal tubular segments 2–3 mm in length. In order to ensure that false negative results were not obtained on segments with relatively low protein content per unit length, e.g., the thick ascending limb (12), a minimum of 5- to 10-mm length of each segment type (containing at least 0.3 μg of protein) was used.

The results indicate that GSH transferase activity in the rabbit nephron resides entirely in the proximal convoluted and proximal straight tubules. Studies in rats demonstrate the identity of glutathione transferase B with ligandin (3) and its localization by immunofluorescence to the proximal tubules of the renal cortex (14). Selective poisoning of the proximal tubule of the rat kidney by heavy metals leads to the appearance of ligandin in the urine and suggests that it is localized preferentially in the terminal parts of the proximal tubule in this species (15). Ligandin has not been purifed from rabbit kidney and anti-rat ligandin IgG does not immunologically cross-react with rabbit tissues. In rats, ligandin is the predominant GSH transferase (16). It is not known whether rabbit kidney contains more than one GSH transferase which catalytically reacts with 1-chloro-2,4-dinitrobenzene.

The evidence that GSH transferase plays a role in organic anion transport by the proximal tubules is inferential and includes studies of competitive binding of ligands, such as penicillin, probenecid, and Bromsulphalein, to renal ligandin, and an increase in renal organic anion excretion following induction of ligandin (2). Since organic anion transport by renal tubular cells involves active transport at the level of peritubular or luminal membranes, failure to demonstrate ligandin in plasma membrane fractions of kidney suggests that binding of ligands to GSH is not directly coupled to the active transport processes. Since conjugation to GSH is the first step in the mercapturic acid pathway, GSH transferase may result in formation of GSH adducts.

In the present study, separated renal tubules were incubated *in vitro*. These tubules retain viability for several hours and demonstrate transport activity (17). Since the tubules are fragmented with their lumens communicating with the external medium, substances transported actively or diffusing across both luminal and peritubular surfaces appear in the incubation medium. In the present study intact proximal tubules, which have glutathione transferase activity, form EA-GSH and EA-cysteine which enter the tubular fluid and presumably act on more distal nephron sites. Papillary nephron segments are deficient in this enzyme activity and form significantly less glutathione or other adducts.

Summary. Ligandin is a major renal organic anion-binding protein and is the major glutathione (GSH)-S-transferase in the rat, monkey, and man. GSH transferase activity was measured in isolated segments of the normal rabbit nephron using 1-chloro-2,4-dinitrobenzene as substrate. Enzyme activity was confined to the proximal convoluted and proximal straight tubules and was not detectable in the loops of Henle and collecting tubules.

Incubation of proximal tubules with [^{14}C]ethacrynic acid resulted in formation of glutathione and cysteine adducts. Loops of Henle and collecting tubules formed significantly less glutathione adduct and no other adducts. GSH transferase in the proximal tubule may thus be important *in vivo* in the formation of thiolated adducts by the kidney.

This work was supported by Research Grant 7R01 AM198 22-01 from the National Institutes of Health, and Grants 2019, 5384, and 70228 from the National Institute of Arthritis and Metabolic Diseases.

1. Arias, I. M., Fleischner, G., Kirsch, R., Mishkin, S., and Gatmaitan, Z., *in* "Glutathione: Metabolism and Function" (I. M. Arias and W. Jakoby,

eds.), p. 175. Raven Press, New York (1976).
2. Kirsch, R., Fleischner, G., Kamisaka, K., and Arias, I. M., J. Clin Invest. **55,** 1009 (1975).
3. Habig, W. H., Pabst, M. J., Fleischer, G., Gatmaitan, Z., Arias, I. M., and Jakoby, W. B., Proc. Nat. Acad. Sci. USA **71,** 3879 (1974).
4. Pabst, M. J., Habig, W. H., and Jakoby, W. B., Biochem. Biophys. Res. Commun. **52,** 1123 (1973).
5. Chasseud, L. F., *in* "Glutathione: Metabolism and Function" (I. M. Arias and W. Jakoby, eds.), p. 77. Raven Press, New York (1976).
6. Burg, M., and Green, N., Kidney Int. **4,** 301 (1973).
7. Fine, L. G., Bourgoignie, J. J., Hwang, K. H., and Bricker, N. S., J. Clin. Invest. **58,** 590 (1976).
8. Fine, L. G., and Trizna, W., Amer. J. Physiol. **232,** F383 (1977).
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193,** 265 (1951).
10. Duggan, D. E., and Noll, R. M., Proc. Soc. Exp. Biol. Med. **139,** 762 (1972).
11. Schmidt, U., and Dubach, V. C., Progr. Histochem. Cytochem. **2,** 185 (1971).
12. Imbert, M., Chabardes, D., Montegut, M., Clique, A., and Morel, F., Pfluegers Arch. **354,** 213 (1975).
13. Schlondorff, D., Fine, L. G., Trizna, W., and Weber, H., Abstr. Amer. Soc. Nephrol. **9,** 112 (1976).
14. Fleischner, G., Mishkin, D., Reyes, H., Robbins, J., Levy, A. J., Gatmaitan, A., and Arias, I. M., J. Clin. Invest. **50,** 31a (abstract) (1971).
15. Feinfeld, D. A., Fleischner, G., Goldstein, E. J., Biempica, M., Arias, I. M., and Bourgoignie, J. J., Kidney Int. (in press).
16. Jakoby, W. B., Ketley, J. N., and Habig, W. H., *in* "Glutathione: Metabolism and Function" (I. M. Arias and W. B. Jakoby, eds.), p. 213. Raven Press, New York (1976).
17. Burg, M. B., and Orloff, J., Amer. J. Physiol. **203,** 327 (1962).

Received July 29, 1977. P.S.E.B.M. 1978, Vol. 157.