The Distribution of some Hydrolases in Glomeruli and Tubular Fragments Prepared from Rat Kidney by Zonal Centrifugation

By D. G. TAYLOR, R. G. PRICE AND D. ROBINSON

Department of Biochemistry, Queen Elizabeth College (University of London), Campden Hill, London W.8, U.K.

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1. A collagenase digest of rat kidney cortex was separated into four bands by zonal centrifugation. 2. Two of these bands were shown by light-microscopy to contain glomeruli and tubular fragments, which were free from each other and well separated from other renal material. 3. Protein, *N*-acetyl- β -glucosaminidase, 5'-nucleotidase, L-leucine β -naphthylamidase, leucine aminopeptidase, acid phosphatase and alkaline phosphatase were assayed across the gradient. 4. The greater proportion of these enzyme activities was recovered in the tubular fragments and acid phosphatase was the only enzyme detected in significant amounts in the glomeruli. 5. Tubular fragments and glomeruli were sedimented and multiple forms of β -naphthylamidase, *N*-acetyl- β -glucosaminidase, acid phosphatase and alkaline phosphatase were investigated by starch-gel electrophoresis.

Recent studies on renal enzymology have attempted to localize enzyme activities in distinct regions of the nephric tubule and glomerulus. Information obtained from whole-homogenate and cellular-fractionation studies does not take account of any variation in enzyme activity in different regions of the kidney. Quantitative histochemical procedures involving the exact recognition, microdissection and microassay of regions of the nephron and glomerulus have been described by McCann (1956), Bonting, Pollak, Muehreke & Kark (1958) and Kissane (1961). Only small quantities of material can be prepared by these methods, but attempts at preparing larger amounts of specific regions of the kidney have been limited to the isolation of glomeruli and tubular fragments. Richterich & Franz (1960) described a technique for separating glomeruli from tubular material from a cortical mash involving centrifugation and sieving through a nylon mesh, and studied the distribution of several enzyme activities. Greenspon & Krakower (1950) isolated glomeruli by forcing a cortical mash through a sieve of known mesh size and van Breda Vriesman & Willighaven (1968) digested dog kidney with collagenase before sieving. Collagenase digestion has also been used in the preparation of rabbit renal tubules by Burg & Orloff (1962).

In a preliminary report Taylor, Price & Robinson (1971) described a procedure that allows a large-scale isolation of glomeruli and tubular fragments simultaneously by rate zonal centrifugation of a collagenase digest of rat kidney cortex. In the

present paper, studies on the distribution of isoenzymic forms of a number of enzymes in glomeruli and tubular fragments from rat kidney are reported.

MATERIALS AND METHODS

Preparation of cortical mash. Male Sprague-Dawley rats (200 g) were killed by a blow on the head, and the renal arteries and veins were exposed and clamped. Then 5 ml of a 0.05% (w/v) collagenase [Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.] solution in 0.9% NaCl (adjusted to pH 8.5 with 0.25 M-NaOH) was injected into each renal pelvis. After 10 min the kidneys were dissected out, the renal capsules removed and the cortices separated from the medullae. A known weight (4–16 g) of cortical preparation was then minced to a paste with a razor blade and made up to a 5% (w/v) suspension in the collagenase solution. The suspension was forced through a no. 1 Luer needle and incubated at 25°C with constant stirring. After 1 h the suspension was filtered through two layers of muslin and the filtrate was washed three times by centrifugation at 5000 *g*-min on an MSE 18 ultracentrifuge, the pellet being resuspended each time in 100 ml of 0.9% NaCl (adjusted to pH 7.4 with 0.25 M-NaOH). The final pellet was taken up in 50 ml of 40% (w/w) sucrose solution (pH 7.4).

Zonal centrifugation. The suspension in 40% (w/w) sucrose was fractionated by zonal centrifugation in an MSE type 'A' zonal rotor at 5000 rev./min at 4°C. A continuous density gradient of 40–60% (w/w) sucrose at pH 7.4 was used, with an overlay of 20% (w/w) sucrose and a cushion of 65% (w/w) sucrose. Finally 30 fractions (35 ml) were collected and the contents were characterized by light-microscopy. Glomeruli and tubular fragments were recovered from these fractions by centrifuging in an MSE 18 ultracentrifuge at 500 000 *g*-min, and the resulting

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pellets were suspended in a small volume of 0.45M-sucrose.

Enzyme assays. Alkaline phosphatase (EC 3.1.3.1) was assayed by a procedure adapted from that of Fernley & Walker (1965). A suitably diluted enzyme fraction (1 ml) was added to freshly prepared 0.1 mM-4-methylumbelliferyl phosphate (1 ml) in 0.05 M- Na_2CO_3 - NaHCO_3 buffer, pH 9.8. After incubation at 37°C for 20 min the reaction was terminated by the addition of 3 ml of 1 M-glycine-NaOH buffer, pH 10.4. The fluorescence of the liberated 4-methylumbelliferone was measured in a Locarte LF5 fluorimeter at 440 nm. L-Leucine β -naphthylamidase (EC 3.4.1.1) was assayed by a method adapted from Greenberg (1962). A suitably diluted enzyme fraction (1 ml) was added to a freshly prepared 0.33 mM-L-leucine β -naphthylamide (1 ml) in 0.1 M-sodium phosphate buffer, pH 7.2. After incubating at 37°C for 20 min, the assay was terminated by the addition of 1 M-glycine-NaOH, pH 10.4 (1 ml). The fluorescence of β -naphthylamine was measured at 410 nm with activation at 335 nm. Acid phosphatase (EC 3.1.3.2) was assayed fluorimetrically by the method of Robinson & Willcox (1969) and *N*-acetyl- β -glucosaminidase (EC 3.2.1.30) by the method of Robinson, Price & Dance (1967). 5'-Nucleotidase (EC 3.1.3.5) was assayed by the method of Heppel & Hilmoe (1955) with the assay of the liberated phosphate as described by Allen (1940). Leucine aminopeptidase (EC 3.4.1.1) was assayed by the method of Raab (1966), and cytochrome oxidase (EC 1.9.3.-) as described by Cooperstein & Lazarow (1951).

Starch-gel electrophoresis. Multiple forms of *N*-acetyl- β -glucosaminidase and L-leucine β -naphthylamidase were separated in starch gel at 4°C in 0.04 M-sodium phosphate buffer, pH 6.8, for 3 h at 200 V and 40 mA (Robinson *et al.* 1967). Acid and alkaline phosphatase isoenzymes were separated in 0.04 M-sodium phosphate buffer, pH 8.0. Enzyme activities in the gel were detected by flooding the gel surface with the appropriate substrate, followed by incubation at 37°C for 30 min, and the resulting fluorescent bands were inspected under u.v. light.

Other methods. Protein was measured by the method of Schuel & Schuel (1967) as modified for use with the Technicon AutoAnalyzer with casein as standard. The concentration of sucrose was determined from the refractive index of the solution by means of an Abbe refractometer (Bellingham and Stanley Ltd., London N.15, U.K.).

RESULTS

The separation of a collagenase digest of rat kidney on an 'A' zonal rotor is shown in Plate 1(a). Four major bands are apparent. The outermost band (4), which occurs at 50–51% (w/w) sucrose, contains predominantly decapsulated glomeruli (Plate 1b). The average diameter of the glomeruli was approx. 100 μm . The glomeruli are clearly separated from the band (3) containing principally tubular fragments and cells (Plate 1c), which occurs at 45–49% (w/w) sucrose. Since the homogenate was prepared from the kidney cortex, these fragments are derived mainly from proximal and distal convolutions of the nephric tubules. Of the two

other bands, one contains erythrocytes (2), and the innermost band (1) contains whole cells and cellular debris.

The distribution of protein and several enzyme activities among the fractions obtained from the rotor is shown in Fig. 1. There are four protein peaks (1–4), corresponding to the four bands obtained in the rotor. The specific activity of acid phosphatase in the fractions with the highest content of tubular fragments was approximately equal to that found in the glomeruli. The peak of acid phosphatase activity at the light end of the gradient is due to cellular debris. The distribution of *N*-acetyl- β -glucosaminidase is similar to that of acid phosphatase in that there is high activity in the tubular fragments and the cellular debris, but there is no peak of hexosaminidase activity in the glomerular fraction.

Alkaline phosphatase, L-leucine β -naphthylamidase, leucine aminopeptidase and 5'-nucleotidase all exhibit similar distributions. There are two main peaks of activity corresponding to cellular debris and tubular fragments, but the activity of all these enzymes in the glomerular fraction is low.

Cytochrome oxidase was assayed as a mitochondrial marker enzyme; three peaks of activity are due to cellular debris, tubular fragments and glomeruli, and a fourth peak, found at 43% (w/w) sucrose, indicates that band 2 (Plate 1a) probably contains mitochondria.

The specific activities of the six hydrolytic enzymes in the glomerular and tubular preparations obtained by centrifuging the corresponding zonal fractions are presented in Table 1. The most active enzyme in the glomerular fraction is acid phosphatase, and it is also the only enzyme to show similar specific activity in both the glomeruli and tubular fragments. All the enzymes in Table 1 have a higher specific activity in the tubular fragment than in the homogenate, whereas in the glomerular preparation only acid phosphatase and alkaline phosphatase show significant increases in their specific activities over that of the homogenate. The specific activity of alkaline phosphatase in the glomerular preparation is 50% greater than the homogenate, whereas the tubular preparation shows a fourfold increase. In contrast, the specific activity of acid phosphatase in both the tubular and glomerular preparations is very similar.

The multiple forms of hydrolases were studied by means of starch-gel electrophoresis. Three anodic forms of acid phosphatase were found in kidney homogenates, but the most rapidly migrating form was absent from the glomerular and tubular preparations. Two forms of alkaline phosphatase were present in the kidney homogenate; both were found in the tubular preparation, but the faster form was absent from the glomeruli. Two

EXPLANATION OF PLATE I

Separation of glomeruli and tubular fragments by zonal centrifugation. Experimental details are given in the text. (a) Zones in the 'A' zonal rotor after 45 min at 5000 rev./min; zone 1, cellular debris; zone 2, erythrocytes; zone 3, tubular fragments; zone 4, glomeruli. (b) Glomeruli from zone 4 (final magnification $\times 160$). (c) Tubular fragments from zone 3 (final magnification $\times 160$). Photographs of unstained glomeruli and tubular fragments were obtained with a Zeiss Photomicroscope.

Fig. 1. Distribution of some hydrolases and protein in fractions obtained from a rat kidney collagenase digest by zonal centrifugation. Details of the conditions of zonal centrifugation are given in the text. Specific activities are given as μmol of substrate hydrolysed/h per mg of protein. Enzymes were assayed as described in the Materials and Methods section. (a) $\bullet$, Sucrose gradient (% w/w); $\blacksquare$, protein profile (mg/ml); (b) $\circ$, acid phosphatase; $\bullet$, *N*-acetyl- β -glucosaminidase. (c) $\bullet$, cytochrome oxidase; (d) 'leucine aminopeptidase', with as substrate: $\blacktriangle$, L-leucine β -naphthylamide; and $\triangle$, L-leucine *p*-nitroanilide. (e) $\blacksquare$, alkaline phosphatase; (f) $\square$, 5'-nucleotidase.

Table 1. Activity of rat kidney hydrolases in glomeruli and tubular fragments

Specific activities (μmol of product/h per mg of protein) observed in a typical fractionation are given. Assay and cell-fractionation procedures were as described in the text.

	Specific activity		
	Homogenate	Tubular fragments	Glomeruli
Acid phosphatase	0.89	1.76	1.54
Alkaline phosphatase	0.30	1.26	0.45
L-Leucine β -naphthylamidase	0.97	1.68	0.32
Leucine aminopeptidase	0.46	0.93	0.16
5'-Nucleotidase	2.89	8.66	1.17
<i>N</i> -Acetyl- β -glucosaminidase	0.88	2.25	0.70

forms of *N*-acetyl- β -glucosaminidase could be demonstrated in the kidney (Price & Dance, 1967). The faster form was absent from both the glom-

erular and tubular preparations. L-Leucine β -naphthylamidase was also present in two forms in kidney homogenates, both of which were present in

the tubular preparation, but the faster form was absent from the glomeruli.

DISCUSSION

Zonal rotors have been used successfully for various separations of subcellular components. The low-speed type 'A' rotor has proved of value in the separation of subcellular organelles such as lysosomes and mitochondria (Schuel, Schuel & Unakar, 1968). Johnston, Mathias, Pennington & Ridge (1968) separated diploid and tetraploid nuclei from rat liver, and Mathias, Ridge & Trezona (1969) used the 'A' rotor to isolate different populations of avian erythrocytes. The results presented in this paper suggest that the 'A' rotor can be utilized to separate different regions of the nephron.

The isolation of distinct morphological and physiological regions of the kidney allows a quantitative assessment to be made of the distribution of various kidney enzymes. The distribution of enzymes in the kidney has been studied by using quantitative histochemistry (Mattenheimer, 1967). To date, only glomeruli and tubular fragments have been isolated in large quantities from the kidney. The methods used have allowed the preparation of one at the expense of the other (Greenspon & Krakower, 1950; Burg & Orloff, 1962). Richterich, Schafroth & Franz (1961-62) were able to prepare small quantities of rat kidney glomeruli and tubular fragments simultaneously. The glomeruli obtained by these methods usually include some glomeruli that still have the Bowman's capsule attached, and both the glomerular and the tubular preparations are usually contaminated to a varying degree by each other. The method described above allows the preparation of large quantities of pure decapsulated glomeruli and a fraction rich in tubular fragments and devoid of glomeruli.

The high specific activity of acid phosphatase in the glomerulus (Table 1) agrees well with the findings of Bonting, Tsoodle, de Bruin & Mayron (1960), who studied human kidney, and of Kissane & Hoff (1962) who studied rat kidney. Alkaline phosphatase, 5'-nucleotidase and leucine aminopeptidase activities are known to be associated with the plasma membrane (Goldfischer, Essner & Novikoff, 1964; Coleman & Finean, 1966), and these enzymes had greatest activity in the tubular fragments. *N*-Acetyl- β -glucosaminidase exhibits a bimodal distribution in subcellular fractions of rat kidney (Price & Dance, 1967) and is present in both the lysosomal and the microsomal fractions. *N*-Acetyl- β -glucosaminidase, unlike acid phosphatase, exhibits only low specific activity in the glomerulus. Our finding that there is higher activity of cytochrome oxidase in the tubular fragments

than in the glomerulus is in agreement with that of Schafroth & Richterich (1963).

Differences in the distribution of isoenzymes of lactate dehydrogenase in glomeruli and tubular fragments have been reported by Richterich *et al.* (1961-62). The hydrolases we have studied are also present in the kidney in several isoenzymic forms. No differences were observed between the multiple forms of acid phosphatase or *N*-acetyl- β -glucosaminidase found in glomeruli and tubular fragments. There were two isoenzymic forms of alkaline phosphatase and β -naphthylamidase present in the tubular preparation but the more rapidly migrating forms of both these enzymes were not detected in the glomerulus.

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