Desulphation of dextran sulphate during kidney ultrafiltration

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The renal clearance of [³H]dextran sulphate by the isolated perfused rat kidney was associated with desulphation of the molecule, as demonstrated by ion-exchange and affinity chromatography of material resident in both glomeruli and urine samples. This process also occurred *in vivo*. The molecular size distribution of glomerular dextran sulphate in the perfused kidney was indistinguishable from that in the perfusate, and although urinary material was smaller it remained macromolecular. Sulphatase

activity was not detected in urine or in the perfusate of perfused kidneys, but was detected in glomerular and non-glomerular cortex fractions isolated by a sieving procedure. The identification of significant biochemical changes to dextran sulphate demonstrates that it does not function as an inert transport probe, and supports the concept of cellular involvement in the process of renal charge selectivity.

INTRODUCTION

It has long been assumed that charged polysaccharides such as dextran sulphate behave as inert molecules when processed during renal filtration. This assumption has formed the basis of the understanding of the striking charge selectivity patterns seen with the transglomerular transport of charged dextran probes (Deen et al., 1980). Fractional clearance studies in rats have demonstrated a marked restriction in the transport of ³H-labelled dextran sulphate, compared with its uncharged counterpart, dextran, for a given hydrodynamic size (Chang et al., 1975). This restriction has been interpreted as being due to electrostatic repulsion of the dextran sulphate by the fixed negative charges of the glomerular basement membrane, and more particularly of heparan sulphate (Deen et al., 1980). Neither dextran nor dextran sulphate has been shown to undergo tubular reabsorption (Chang et al., 1975) or depolymerization to small ³H-labelled fractions as studied by gel chromatography.

More recently, the electrostatic basis for the differential transport of dextran sulphate as compared with dextran has been questioned (Zamparo and Comper, 1990). Charge selectivity cannot be mimicked with isolated GBM preparations (Bray and Robinson, 1984; Bertolatus and Klinzman, 1991) and the GBM anion charge is much lower than that proposed to be required to exert charge repulsion (Comper et al., 1993). Rather, glomerular cell processing, possibly through endothelial cell uptake and release of dextran sulphate but not dextran, appears to be intimately involved in the renal processing leading to charge selectivity (Tay et al., 1991).

The present study shows that dextran sulphate is indeed modified biochemically during ultrafiltration, with significant desulphation of the ³H-labelled material that appears in the urine both *in vivo* and in isolated perfused kidneys. These results support the concept of renal cell involvement in the processing of dextran sulphate.

EXPERIMENTAL

Materials

Male Sprague–Dawley rats (300–350 g) were obtained from Monash University and Sydney University Central Animal

Houses. The dextran sulphate preparation, with a weight average M_r of approx. 42 000 (batch no. Op 321525) (average 1.7 sulphate groups per glucose residue), Sephadex G-100, Sepharose Q and Sepharose CL-4B were from Pharmacia, Uppsala, Sweden. Polybrene, CHAPS and Tris were from Sigma Chemical Co., St. Louis, MO, U.S.A. Urea was from Ajax Chemicals, Clyde Industries, Australia. Sodium borof[³H]hydride (132 mCi/mg) was from Amersham International. A disaccharide derived from heparin, α -L-iduronic acid 2-sulphate (1,4)-anhydro[1-³H]-mannitol 6-sulphate (formula M_r 498; one sulphate group per saccharide) and a tritiated pentasaccharide derived from chondroitin 6-sulphate (formula M_r 1252; 0.6 sulphate group per saccharide) were generously provided by Dr. J. J. Hopwood (Adelaide Children's Hospital). Papain was generously donated by Dr. H. C. Robinson (Department of Biochemistry, Monash University). All other reagents used were of analytical standard.

Chemical methods

Tritiation of dextran sulphate was carried out by a sodium borof[³H]hydride reduction procedure as described by Van Damme et al. (1982). The sample had a specific radioactivity of 4.26×10^7 d.p.m./mg. Polybrene–Sephadex was prepared using a 2% solution of Polybrene according to the method of Dawes et al. (1985).

Chromatographic analysis of radiolabelled material

Samples were analysed by affinity chromatography on a 5 cm $\times$ 5 cm² column of Polybrene–Sephadex. The samples were applied in 19 mM citrate buffer, pH 6.0, and eluted with a linear gradient of 0–3 M NaCl in the same buffer at a flow rate of 0.5 ml/min as described in detail by Dawes (1988). The recoveries using this technique were 95–105%. Alternatively, samples were subjected to ion-exchange chromatography using a 19 cm $\times$ 1 cm² column of Sepharose Q. The samples were applied in 6 M urea, 0.05 M Tris, 0.05% (w/v) CHAPS, pH 7.0 and eluted with a linear gradient of 0.15–2.5 M NaCl in the same buffer at a flow rate of 0.5 ml/min. The recoveries using this technique were in the range 100–105%. Size-exclusion chromatography was performed on a 25 cm $\times$ 1.0 cm² Sephadex G-100 column as described by Tay et al. (1991).

Abbreviations used: GBM, glomerular basement membrane; IPK, isolated perfused kidney.

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Kidney perfusion *ex situ*

The perfusion of kidneys from 300–350 g Sprague–Dawley rats for up to 1.5 h with Krebs–Henseleit buffer containing 5% BSA and amino acids has been described previously (Tay et al., 1991). The perfusate contained dextran sulphate at 15 µg/ml.

In vivo processing of [³H]dextran sulphate

Sprague–Dawley rats maintained in a metabolic cage were injected with 1 mg of [³H]dextran sulphate in the metatarsal vein. This gave an initial plasma concentration of 80 µg/ml, which was rapidly cleared. It was considered to be comparable with the concentration used for the isolated perfused kidney of 15 µg/ml. At 2, 3 and 24 h after injection, blood samples were collected by cardiac puncture; urine samples were taken at 2, 6 and 24 h. These samples were then analysed by affinity chromatography.

Preparation of glomeruli

Glomeruli were isolated by the sieve method described by Spiro (1967) and suspended in 5 ml of 0.15 M NaCl on ice. After adequate mixing, a 20 µl sample of the suspension was taken up into a calibrated micro-capillary tube and the glomeruli were counted under the phase-contrast microscope. Samples (1 ml), containing a known number of glomeruli, were incubated with 0.15 ml of papain buffer (1.0 M sodium acetate, pH 5.5, with 0.05 M EDTA) containing 10 mg/ml cysteine hydrochloride (added immediately before use) and 6.25 µl of papain suspension (16 µg/ml) at 60 °C for 24 h.

Counting of radioactivity

³H radioactivity was determined in 1.0 ml aqueous samples with 9.0 ml of scintillant as described by Fox (1968) and recorded on an LKB–Wallac 1215 Rackbeta scintillation counter.

RESULTS

Affinity chromatography on Polybrene–Sephacrose of the initial perfusate of an isolated perfused kidney is compared with that of labelled material in the glomeruli and in a urine sample collected between 1 h and 1 h 20 min in Figure 1. The bulk of the [³H]dextran sulphate in the perfusate eluted at a concentration of 1.8 M NaCl. By contrast, two peaks of labelled glomerular material were identified; one eluted at a lower salt concentration than the starting material (1.65 M NaCl) and the other did not bind at all. Most of the labelled material in the urine sample collected between 1 h and 1 h 20 min had no affinity for Polybrene and the remainder eluted in a broad peak over the range 0.3–1.45 M NaCl. The failure of much of this material to bind to Polybrene was not due to degradation into small oligosaccharides. A sulphated disaccharide prepared from heparin and a chondroitin 6-sulphate pentasaccharide, both of which were less highly sulphated than the dextran sulphate used in this study, bound to Polybrene under these conditions and eluted at 0.15 and 0.3 M NaCl respectively. The results from three experiments with isolated perfused kidneys are summarized quantitatively in Table 1, giving the percentages of the material that were unmodified (high binding affinity), had reduced affinity for the column, and remained unbound. These data are compared with results from a single experiment in which samples were analysed by ion-exchange chromatography on Sepharose Q. They demonstrate that in the isolated perfused kidney the [³H]dextran sulphate in the perfusate appears to be essentially

unchanged during the course of the 1 h 20 min perfusion, and confirm that material in both the glomerular and urine samples has either reduced or no affinity for the column.

Approximately 1% of the dextran sulphate in the perfusate appeared in the urine after 1 h of perfusion. To address the possibility that this might constitute, in part, the small fraction

Figure 1 Affinity chromatography on Polybrene–Sephacrose of [³H]dextran sulphate in perfusate, glomeruli and urine samples from the isolated perfused kidney

Samples were eluted from a Polybrene–Sephacrose affinity column with a linear 0–3.0 M NaCl gradient as described in the Experimental section. Perfusate (□) was collected at the beginning of the perfusion and urine samples (○) were collected between 1 h and 1 h 20 min. Glomeruli (●) isolated from the perfused kidney were digested with papain before the samples were applied to the column. The straight line represents the NaCl gradient.

Table 1 Distribution of [³H]dextran sulphate samples on a Polybrene affinity column

Data are given as means ± S.D. (*n* = 3). For the isolated perfused kidney (IPK), the final perfusate sample was taken at 1 h and the urine samples between 1 h and 1 h 20 min. During *in vivo* experiments plasma was taken at 2, 3 and 24 h and urine at 2, 6 and 24 h after intravenous injection of [³H]dextran sulphate. There was no evidence of a trend in the desulphation pattern with time and the results have been pooled. A comparison of results obtained on Sepharose Q columns using different animals is shown in parentheses.

Sample	Content (%)		
	Unbound	Reduced affinity	Unmodified
Dextran sulphate control	6.2	0	93.8
IPK (1 h)			
Perfusate (initial)	8.4 ± 4.6 (7.2)	0 (0)	91.6 ± 4.6 (92.8)
Perfusate (final)	11.1 ± 5.1	0	88.9 ± 5.1
Urine (1 h–1 h 20 min)	67.2 ± 18.1 (59.6)	31.6 ± 19.9 (27.5)	1.8 ± 2.5 (13)
Glomeruli (1 h 20 min)	26.5 ± 14.0	70.4 ± 13.1	3.2 ± 1.1
Whole rat			
Plasma (2–24 h)	78.0 ± 4.5	9.0 ± 1.4	12.9 ± 1.8
Urine (2–24 h)	73.5 ± 11.1	17.7 ± 6.9	9.4 ± 4.8

Figure 2 Size-exclusion chromatography on Sephadex G-100 of [^3H]dextran sulphate in perfusate, glomeruli and urine samples from the isolated perfused kidney

Perfusate (□) was collected at the beginning of the perfusion and urine samples (○) were collected between 1 h and 1 h 20 min. Glomeruli (●) isolated from the perfused kidney were digested with papain before the samples were applied to the column. V_0 indicates the void volume and V_t the total volume of the column.

of the starting material that had no affinity for Polybrene or Sepharose Q, a dextran sulphate sample from which this material had been eliminated by affinity chromatography was prepared. When this fraction was used in the isolated perfused kidney under conditions identical to those used previously, the results obtained from two experiments analysed on Sepharose Q were $17.2 \pm 1.4\%$ unbound, $28.8 \pm 0.8\%$ with reduced affinity and $53.9 \pm 2.0\%$ unmodified. These results confirm that nearly 50% of the high-affinity fraction of dextran sulphate is modified during ultrafiltration.

Size-exclusion chromatography of perfusate samples showed that the molecular size distribution of unpurified dextran sulphate in the perfusate remained constant during the experimental period (results not shown). The molecular size distribution of labelled material in the glomerular fraction closely resembled that in the perfusate. Urinary material was smaller, as expected, since the isolated perfused kidney exhibits normal size selectivity (Tay et al., 1991). There was no evidence of any significant depolymerization of the urinary dextran sulphate, as $> 85\%$ remained essentially macromolecular (Figure 2).

These results suggest that desulphation of the dextran sulphate had occurred. This is particularly indicated by the *de novo* appearance of material with reduced affinity for the ion-exchange column. These fractions were not the result of artifactual adsorption of dextran to the column, as analysis of dextran fractions of similar molecular weight revealed that there was no binding to either Sepharose Q or Polybrene (results not shown). The results also suggest that this desulphation process is localized, in part, to the glomeruli.

In a separate experiment, we tested for sulphatase activity in the urine sample by incubating urine collected from an isolated perfused kidney in the perfusion period of 1 h to 1 h 20 min with added [^3H]dextran sulphate at 37°C for 90 min. The mixture was

then applied to the affinity column. No change in the profile was apparent (results not shown), indicating that no desulphation occurs in the urine.

We also tested for sulphatase activity in glomerular and non-glomerular cortex fractions separated by the sieving procedure (see the Experimental section) using a purified labelled dextran sulphate. Approximately 12000 glomeruli were incubated with 4.2 ml of $15 \mu\text{g/ml}$ [^3H]dextran sulphate for 2 h in Krebs-Henseleit buffer containing 5% BSA with amino acids. The non-glomerular fractions isolated from single kidneys were incubated under similar conditions, but without the BSA. After the incubations the material was pelleted, digested with papain and then centrifuged. The supernatants (combined from three separate experiments for both glomerular and non-glomerular fractions) were then applied to a Sepharose Q column and the following results were obtained: glomerular fraction, 6.3% unbound, 13.3% reduced affinity and 80.4% unmodified; non-glomerular fraction, 24.7% unbound, 14.6% reduced affinity and 60.7% unmodified (the original preparation was 100% unmodified). These results demonstrate that sulphatase activity exists in the various isolated cortex fractions, although it remains to be determined which activity is primarily responsible for the changes observed to material appearing in the urine.

To determine whether this modification of dextran sulphate also occurs *in vivo*, the same unpurified [^3H]dextran sulphate sample was injected intravenously into rats. An initial plasma concentration of $80 \mu\text{g/ml}$ gave sufficient radioactivity to permit analysis of the samples. This dextran sulphate was rapidly cleared. Plasma was taken at 2, 3 and 24 h and urine at 2, 6 and 24 h, and selected samples were analysed by affinity chromatography on Polybrene; the results are given in Table 1. The dextran sulphate in the urine was significantly desulphated, as in the urine samples obtained from the isolated perfused kidney. Most of the radiolabelled material in the plasma was also desulphated within 2 h, which contrasts with the IPK perfusate. While the glomerular fractions were not analysed by affinity chromatography, we did identify considerable amounts of dextran sulphate in them; at 2, 3 and 24 h there were 9.01, 14.33, 10.50 ng of dextran sulphate respectively per 1000 glomeruli (the amounts of dextran sulphate were calculated assuming the same specific radioactivity as the injected material).

DISCUSSION

The central observation of this study is that when [^3H]dextran sulphate supplied in the perfusate of an isolated perfused kidney system was subsequently isolated from glomeruli and urine, it no longer retained its original high affinity for Polybrene. Both isolates contained material with reduced affinity, and in both a significant percentage of the radiolabel was unbound. We interpret this result to indicate that dextran sulphate undergoes desulphation in the kidney.

Alternative interpretations, e.g. that the dextran sulphate may be subjected to major degradation such that the products no longer bind to Polybrene, or that the kidney selectively takes up and filters the small fraction of the original material with no affinity for Polybrene, were addressed by further experiments. Size-exclusion chromatography of the glomerular material showed that its molecular size distribution was indistinguishable from that of the [^3H]dextran sulphate in the perfusate, and that although the urinary material was smaller most of it remained macromolecular. Moreover, although the affinity of sulphated polysaccharides for Polybrene does have a size-dependent component (Dawes, 1988), di- and penta-saccharides which were derived from heparin and chondroitin sulphate, and had a lower

sulphate content than the dextran sulphate preparation used here, both bound to Polybrene. The unbound material identified in the glomeruli and the urine could not therefore be small sulphated degradation products. Anion-exchange chromatography confirmed this conclusion, for as the sulphate residues are the only charged groups on dextran sulphate, only complete desulphation will prevent its interacting with such a matrix.

We also demonstrated that the kidney did not appear to selectively take up and filter that fraction of the original material with no affinity for Polybrene or Sepharose Q. This material was removed by affinity chromatography, and when the fraction with high affinity for Sepharose Q was administered in the perfusate of the isolated perfused kidney system, the results indicated that there was modification to nearly 50% of initial material.

The possibility of the dextran sulphate combining as a tight complex with plasma or perfusate proteins or urine components to register altered binding to the ion-exchange column seems insignificant as perfusate samples ran normally (without evidence of desulphation), as did control incubations of dextran sulphate mixed with urine.

Sulphated polysaccharides have been shown to undergo endocytosis by endothelial cells (Bârzu et al., 1985; Hiebert and McDuffie, 1989) and the observation of catabolism of dextran sulphate by desulphation is consistent with other studies. A similar catabolic mechanism has been suggested for the naturally occurring sulphated polysaccharides heparin (Dawes and Pepper, 1979) and heparan sulphate (Dawes and Pepper, 1992) as well as for the synthetic pentosan polysulphate (MacGregor et al., 1984; Dawes and Pepper, 1992). The mechanism for the removal of sulphate residues does not appear to be specific for the type of sulphated polysaccharide. We were unable to demonstrate sulphatase activity in either the urine or the perfusate of perfused kidneys, although sulphatase activity was present in glomerular and non-glomerular cortex fractions. We suggest that both glomerular and tubular sulphatases may participate in the changes to dextran sulphate.

Nevertheless, the use of dextran sulphate to study charge selectivity in kidney ultrafiltration has relied on its supposed properties as an inert transport probe, and there has been no

previous attempt to compare its charge density in plasma and urine fractions. We found that major desulphation also occurs *in vivo*, though in contrast to the perfusate in the isolated perfused kidney system, desulphated material also appears in the plasma and the role of the kidney is therefore more difficult to delineate. These observations put into question the significance of the *in vivo* fractional clearance data for dextran sulphate (Chang et al., 1975), as desulphation occurring before or during filtration will change the size and shape of the molecule. Clearly, dextran sulphate is not an inert transport probe, and the interpretation of studies in which it has been used to examine charge selectivity needs to be reassessed.

This work was supported by grants from the National Health and Medical Research Council of Australia to W.D.C. and from the Australian Research Council to J.D. We gratefully acknowledge the expert technical assistance of Mrs. Lynette Pratt and Ms. Jane Taylor, and gifts of papain from Dr. H. C. Robinson (Department of Biochemistry, Monash University) and tritiated sulphated di- and penta-saccharides from Dr. J. J. Hopwood (Adelaide Children's Hospital).

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