Biochimica et Biophysica Acta, 339 (1974) 92–102

BBA 76554

THE IN VIVO LOCALIZATION OF HIGH-AFFINITY PHLORIZIN RECEPTORS TO THE BRUSH BORDER SURFACE OF THE PROXIMAL TUBULE IN DOG KIDNEY*

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(Received September 18th, 1973)

SUMMARY

The interaction of [^{3}H]phlorizin (spec. act. 5 Ci/mmole) with canine renal tubular surfaces has been investigated in vivo, using the multiple indicator dilution technique.

1. Over short observation periods there is no observable interaction between tracer phlorizin and the antiluminal (peritubular) surface. Instead, phlorizin appears to have a limited volume of distribution relative to the extracellular reference (creatinine), from the postglomerular circulation.

2. Phlorizin and creatinine are filtered to the same degree at the glomerulus however the urine recovery of [^{3}H]phlorizin is 5–10% of the glomerular marker (plasma glucose concentration 60–124 mg/100 ml).

3. The remaining 90–95% can be "washed off" and recovered in the urine within minutes after the systemic administration of a pulse of unlabelled phlorizin or glucose. Preloading of animals with phlorizin or glucose also increases the urine recovery of [^{3}H]phlorizin relative to control conditions. In contrast the urine recovery of [^{3}H]phlorizin is unaffected by phloretin infusion.

4. It is concluded that only one set of high-affinity phlorizin receptors is present in dog kidney, and that these are located along the brush border of the proximal tubule, in close association with the glucose-transport mechanism.

INTRODUCTION

The results of earlier studies on the specificity of sugar reabsorption across the luminal membrane [1, 2] point to the existence of two and possibly three sets of membrane receptors for monosaccharides. Of the 23 pyranosides tested, only 8 have any observable interaction with the brush border of the proximal tubule. Seven of

* A portion of this work was presented in preliminary form at the American Physiological Society August 1973.

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these eight pyranosides share a phlorizon-sensitive receptor with D-glucose. Thus the phlorizin binding sites along the brush border of the proximal tubule must be closely associated if not identical with the glucose transport mechanism. In these experiments the localization of phlorizin inhibition of glucose transport at the luminal membrane was deduced from simultaneous renal vein and urine outflow curves obtained in response to a pulse injection into the renal artery. The present study was undertaken to demonstrate in a more direct fashion phlorizin binding to the brush border surface. The intrarenal distribution of radiolabelled phlorizin in dog kidney was determined in vivo using the multiple indicator dilution technique. Since our methodology permits us to distinguish luminal from antiluminal events [3], we were, therefore, able to examine the interaction of phlorizin with the peritubular membrane as well as the brush border.

90–95% of a tracer dose of filtered phlorizin is bound to the luminal membrane. All of the [^{3}H]phlorizin which is bound to the brush border can be "washed off" by a systemic loading dose of either unlabelled phlorizin or D-glucose. There is no detectable "wash off" of bound [^{3}H]phlorizin in response to a loading dose with phloretin. These data provide strong evidence for the existence of a homogeneous set of stereospecific membrane sites localized to the brush border of the proximal tubule which have a high affinity for phlorizin and are close to, if not identical with the glucose-transport receptor.

In contrast, [^{3}H]phlorizin has no observable interaction with the antiluminal surface of the nephron. This finding does not exclude a slow uptake of phlorizin across the antiluminal membrane but does suggest that any phlorizin receptors imbedded in the peritubular membrane must be low affinity sites compared to those imbedded in the luminal surface.

MATERIALS AND METHODS

The basic experimental surgical and analytical methods used in these studies have been described in previous publications [3, 4]. ^{3}H-labelled phlorizin was obtained from New England Nuclear with a specific activity of 5 Ci/mmole. The radiopurity of this compound was verified by thin-layer chromatography.

RESULTS

Fig. 1 shows the renal vein and urine transit patterns for T1824 (vascular reference), creatinine (extracellular reference), and [^{3}H]phlorizin. Table I shows the data for renal vein recoveries and mean transit times along with the simultaneous urine recoveries of creatinine and phlorizin. All calculations have been performed as previously described [5].

Renal vein data

As shown elesewhere [1, 3], the renal vein outflow data provide information about the interaction of test solutes with the antiluminal surface of the nephron. Inspection of Fig. 1 shows that phlorizin emerges in the renal vein ahead of creatinine, peaks earlier, and is slightly lower on the downslope. This apparent precession of phlorizin over the reference marker creatinine could be due to (i) phlorizin binding to

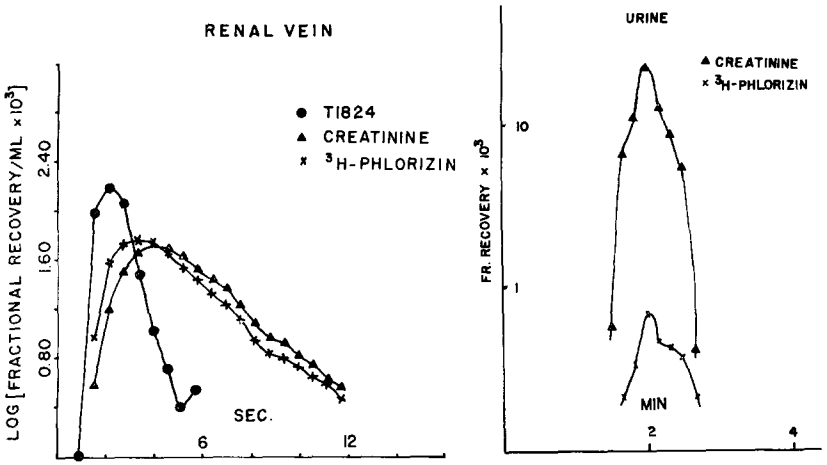

Fig. 1. Renal vein and urine outflow patterns for T1824 (●), creatinine (▲) and [^{3}H]phlorizin (×). The renal vein outflow data have been corrected for the effects of catheter distortion [19].

a compound in plasma i.e. albumin, (ii) phlorizin binding to red cells, or (iii) decreased permeability of the postglomerular capillary to phlorizin relative to creatinine. Moreover if the observed phlorizin precession is the result of binding to a component of whole blood, then this interaction must occur within 1–2 s in order to be of significance in our studies.

To rule out (i), two injection solutions were prepared. In the first, the reference indicators (T1824 and creatinine) along with radiolabelled phlorizin were dissolved in 0.9% NaCl. In the second, the indicators were mixed in fresh dog plasma and equilibrated for about 1 h at room temperature. If a significant fraction of tracer phlorizin binds tightly to a component of plasma, then we expect that the renal vein outflow curve for tritium using the "plasma" injection solution should have two peaks, the first coinciding in time with the emergence of T1824–albumin, and the second emerging with creatinine. When the experiment was performed a double-humped curve was not observed.

Although phloretin has marked affinity for human red cells, phlorizin exhibits negligible binding under the same conditions [6]. Also the half time of equilibration of isotopic glucose and galactose with dog red cells is of the order of 4 h [7, 8]. Therefore it seems unlikely that the glucose transport system is well developed in dog erythrocytes and we would not expect significant phlorizin binding to red cells in the present studies.To test this supposition experimentally the renal vein curves for T1824, creatinine, and [^{3}H]phlorizin were compared in the same animal as a function of decreasing hematocrit. If significant binding occurs to red cells the precession phenomenon should disappear as the hematocrit is lowered [9]. The results are shown in Fig. 2. For hematocrits ranging from 40–19%, the relative outflow curves for phlorizin and creatinine remain unchanged.

In another type of experiment, known amounts of [^{3}H]phlorizin were added to 1-ml samples of heparinized dog blood of varying hematocrit. The samples were spun and an aliquot of the supernatant was removed for counting in a liquid scintillation spectrometer. The results are shown in Table II. If a tracer substance has an

TABLE I

INDICATOR DILUTION DATA

Date	Dog Wt (kg)	Hematocrit (%)	Plasma glucose (mg/100 ml)	Renal vein				Urine			
				$\Sigma CR^{\star}$ / $\Sigma T1824$	$\Sigma^3H^{\star}$ / $\Sigma T1824$	$\bar{t}_{CR}^{\star\star}$	$\bar{t}_{3_H}^{\star\star}$	Total collection time (min)	ΣCR	Σ^3H	$1-\frac{\Sigma^3H^{\star\star\star}}{\Sigma CR}$
Jan. 8/73	13	37	118	0.67	0.70	6.1	6.3	5	0.07	0.005	0.93
	13	38	124	0.63	0.67	5.5	6.3	5	–	–	–
Jan 22/73	17	39	96	0.76	0.83	5.9	5.6	5	0.07	0.005	0.93
April 30/73	15	28	73	0.78	0.82	5.5	5.2	10	0.19	0.01	0.95
	15	28	85	0.87	0.89	4.7	4.6	–	–	–	–
May 8/73	17.5	50	60	0.68	0.76	5.6	5.2	–	–	–	–
May 16/73	17.0	51	84	0.72	0.70	5.4	4.9	–	–	–	–
June 5/73	14.5	34	86	–	–	–	–	10	0.13	0.02	0.85
July 9/73	13.2	34	78	0.96	0.90	9.7	9.0	–	–	–	–
	13.2	19	76	0.95	0.89	5.4	4.7	–	–	–	–
July 19/73	18	31	72	0.88	0.80	4.9	4.5	15	0.21	0.04	0.81

$\star$ $\Sigma CR/T1824$ and $\Sigma^3H/T1824$ represent the renal vein recoveries of creatinine and labelled phlorizin relative to that of T1824.

$\star\star$ $\bar{t}_{CR}$ and $\bar{t}_{3_H}$ represent the renal vein mean transit times of creatinine and phlorizin respectively. These values have been corrected for catheter mean transit time.

$\star\star\star$ $1-\Sigma^3H/\Sigma CR$ represents the "fraction" of isotope reabsorbed, or in the case of [^{3}H]phlorizin, the fraction bound to the luminal membrane

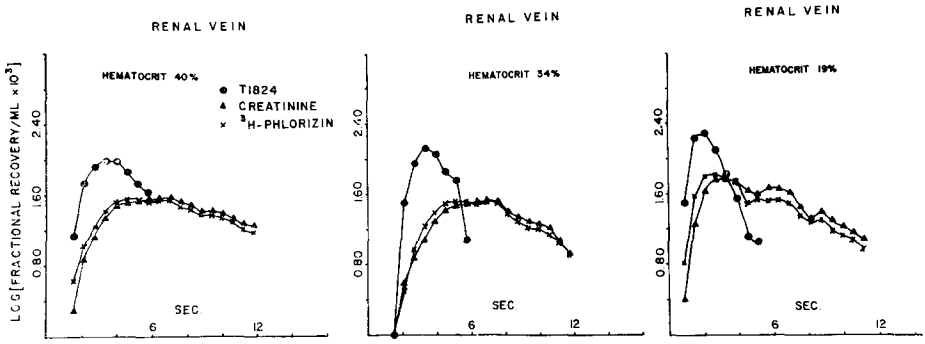

Fig. 2. The renal vein outflow curves for T1824 (●), creatinine (▲), and [^{3}H]phlorizin (×), as the hematocrit is decreased from 40 to 19 %. All curves have been corrected for catheter distortion. The precession of phlorizin over creatinine is not affected by variation in hematocrit.

TABLE II

IN VITRO DISTRIBUTION OF [^{3}H]PHLORIZIN IN DOG BLOOD

The same amount of [^{3}H]phlorizin was added to 1-ml samples of fresh heparinized dog blood. The individual samples were then spun and an aliquot of the supernatant plasma was removed for radioactivity determination. Values show the mean of two determinations for each hematocrit. Total added radioactivity is assumed equal to the plasma recovery for the O-hematocrit sample.

Hematocrit (%)	Radioactivity in sample aliquot (cpm)	Plasma recovery of radioactivity (cpm × (1 − hematocrit))	Plasma recovery [^{3}H]phlorizin (cpm) / Total added radioactivity (cpm)
0	6485	6485	1.00
10	7223	6500	1.00
20	8008	6406	0.99
30	8936	6255	0.96
40	10552	6334	0.98
50	11066	5533	0.85

extracellular distribution we expect its concentration in the plasma phase to increase as the hematocrit increases. Inspection of Table II shows this to be true for [^{3}H]-phlorizin. Calculation of the relative recovery of [^{3}H]phlorizin in the plasma supernatant compared to the amount added shows that all of the label is recoverable in the extracellular fluid (Table II), (within the limits of error introduced by comparing pipettings of high hematocrit samples).

Aside from the negative results quoted above there are two other observations which suggest that the precession of phlorizin over creatinine can not be explained on the basis of binding to a component of whole blood. If such binding did in fact occur, then the renal vein recoveries of [^{3}H]phlorizin relative to T1824 should be consistently greater than creatinine. Inspection of Table I reveals that this is not true. Also, it will be shown below that all of the tracer phlorizin which binds to the luminal surface can be recovered in the urine following a systemic flushing dose of "cold" phlorizin (if the urine collection is carried over a sufficiently long period). Under these loading

conditions the total urine recovery of [^{3}H]phlorizin becomes equal to that of simultaneously filtered creatinine (the glomerular marker). We conclude that [^{3}H]phlorizin and creatinine are filtered equally at the glomerulus and that there has been no significant binding of the drug to any constituent of dog whole blood.

Chinard [10] has pointed out that inulin and creatinine behave identically as glomerular markers, but that there is a limited "volume of distribution" of inulin compared to creatinine from the postglomerular capillary circulation. He proposed that this limited volume could reflect either a decreased permeability of the peritubular capillaries or an exclusion volume effect. In Fig. 3 we show the relative renal vein outflow curves for T1824, creatinine, [^{14}C]inulin and [^{3}H]phlorizin. Inspection shows that the indicators appear in the order of decreasing size: T1824–albumin, inulin, phlorizin and creatinine. Thus the results illustrated in Fig. 3 indicate that the "apparent" volume of distribution available for inulin, phlorizin and creatinine from the postglomerular circulation is a function of their relative sizes and is consistent with Chinard's hypothesis. The apparent limited extracellular volume of distribution observed in our experiments implies that during our observation period there is no demonstrable interaction of phlorizin with the antiluminal surface. This finding by itself does not completely exclude the possibility that such an interaction exists. Most of the [^{3}H]phlorizin is distributed extracellularly. However the fact that the renal vein recovery of [^{3}H]phlorizin is not significantly less than creatinine (Table I) argues

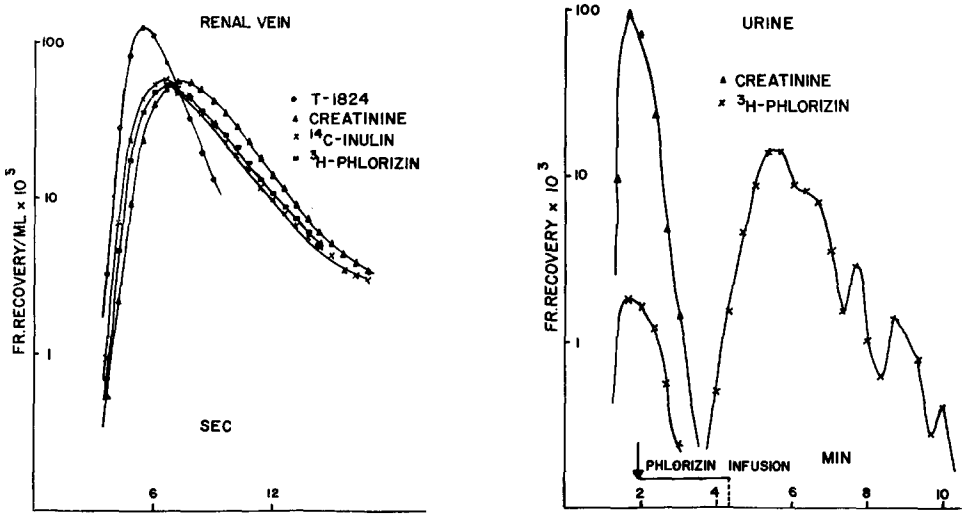

Fig. 3. Simultaneous renal vein transit patterns for T1824 (●), creatinine (▲), [^{14}C]inulin (×), and [^{3}H]phlorizin (■). This experiment demonstrates that the initial upslope is a function of the molecular size of the indicator. The greater the molecular weight, the steeper the upslope. These curves are uncorrected for catheter distortion.

Fig. 4. A pulse injection of a mixture of indicators was made into the left renal artery and the urine outflow curves were determined for creatinine (▲), and [^{3}H]phlorizin (×). 2 min after the injection, 500 mg of phlorizin (in 20 mM $NaHCO_3$) was infused rapidly into the femoral vein over a period of 2–3 min. Following the administration of phlorizin a pulse of ^{3}H radiolabel appears in the urine. There is no concomittant increase in creatinine. Thus the [^{3}H]phlorizin appears to have been washed-off the surface of the luminal membrane.

against the existence of a high affinity transport system at the antiluminal surface capable of extracting the tracer compound.

Urine data

Inspection of Fig. 1 reveals that only small amounts of [^{3}H]phlorizin are recovered in urine. Assuming that phlorizin is filtered equally along with creatinine at the glomerulus, then the fractional extraction of tracer phlorizin by the luminal surface of the nephron is 90–95% of the amount filtered. This apparent binding of tracer phlorizin to the luminal surface of the nephron was further investigated by carrying out studies with systemic loading of phlorizin, D-glucose, and phloretin. Tables III and IV show the urine recovery data for these loading experiments.

TABLE III

EFFECT OF PHLORIZIN AND GLUCOSE PRELOADING

Glucose and phlorizin were administered 15–30 min before intraarterial injection of [^{3}H]phlorizin. $1-\Sigma^3H/\Sigma CR$ represents "bound" tracer phlorizin. The values in Table III should be compared to those in Table I in the absence of preloading.

Date	Loading drug	ΣCR	Σ^3H	$1-\frac{\Sigma^3H}{\Sigma CR}$
Jan 8/73	phlorizin (100 mg)	0.18	0.09	0.43
Jan 22/73	glucose (plasma concn 514 mg/100 ml)	0.17	0.06	0.64

TABLE IV

URINE "WASH OUT" OF LUMINAL BOUND [^{3}H]PHLORIZIN FOLLOWING INFUSION OF GLUCOSE, PHLORIZIN, AND PHLORETIN

A pulse of [^{3}H]phlorizin was given into the left renal artery. 2–4 min later a systemic infusion of the appropriate drug was given over a further 2–5 min. Estimated bound phlorizin is calculated from the urine recovery in the first 4 min. The "washed off" fraction is calculated from the urine recovery in response to the infused drug.

Date	Total collection time (min)	Bound phlorizin	Washed off fraction
April 30	10	0.98	0.39 (plasma glucose 506 mg/100 ml)
May 8	10	0.98	0.55 (Phlorizin 500 mg)
May 16	10	0.98	0.08 (phloretin 500 mg)
July 19	25	0.82	0.82 (phlorizin 200 mg)

Inspection of Figs 4 and 5 reveals that phlorizin and glucose loading successfully "wash-off" almost all of the phlorizin bound to the luminal surface. If the loading dose is administered prior to the experimental run, then unlabelled phlorizin and glucose each have the effect of inhibiting binding of [^{3}H]phlorizin (Fig. 6). In contrast to the effects of phlorizin and glucose, systemic loading with phloretin, the aglycone of phlorizin, does not result in any significant "wash-off" of luminal-bound isotope (Fig. 7).

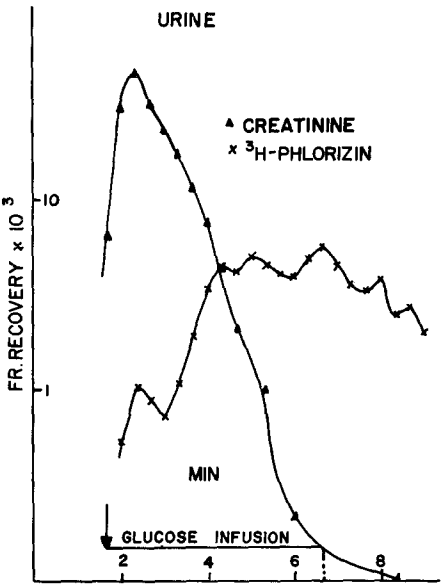

Fig. 5. The urine outflow curves for creatinine (▲), and [^{3}H]phlorizin (×). The same experimental protocol was repeated as shown in Fig. 4, but this time, glucose (50 g) was rapidly infused instead of unlabelled phlorizin. Once again there is an apparent wash-out of ^{3}H label.

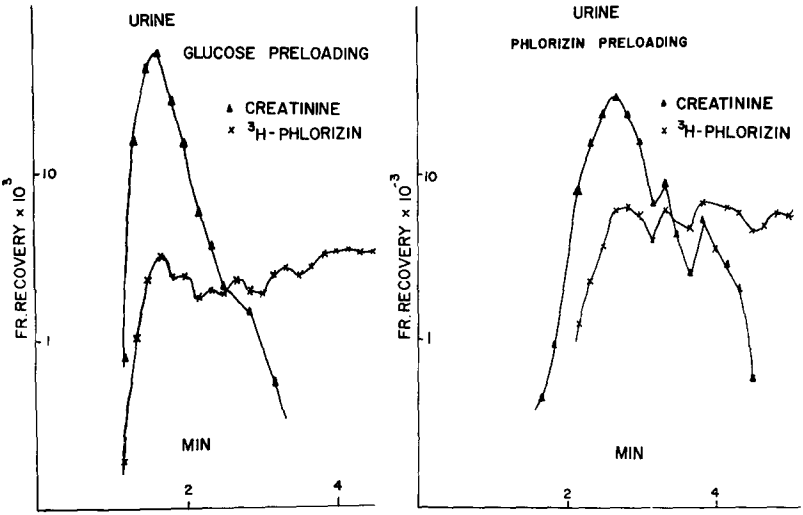

Fig. 6. Shows urine outflow curves for creatinine (▲), and [^{3}H]phlorizin (×). In these experiments, the animals were preloaded with glucose (left hand panel), or phlorizin (right hand panel). In the glucose preloading, a plasma glucose concentration of 514 mg/100 ml was reached at the time of the experiment. Phlorizin preloading was accomplished by intravenous injection of 100 mg phlorizin 15–30 min before the experimental run. The plasma glucose concentration in the phlorizin preloading was 124 mg/100 ml.

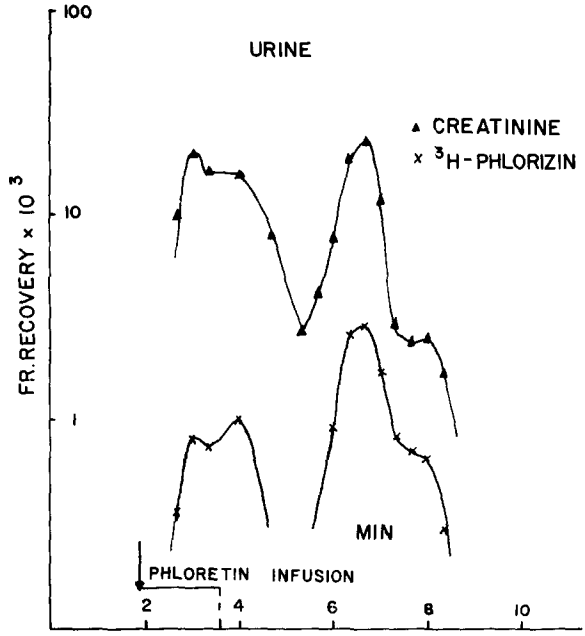

Fig. 7. Urine transit patterns for creatinine (▲), and [^{3}H]phlorizin (×). The same protocol as obtained in Figs 4 and 5 was repeated in an attempt to wash off [^{3}H]phlorizin by a rapid systemic bolus of phloretin. The drug caused a transient drop in blood pressure which resulted in bumpy urine outflow curves. It is evident however, that the administration of phloretin does not result in any significant wash off of [^{3}H]phlorizin from the luminal membrane (see Table IV).

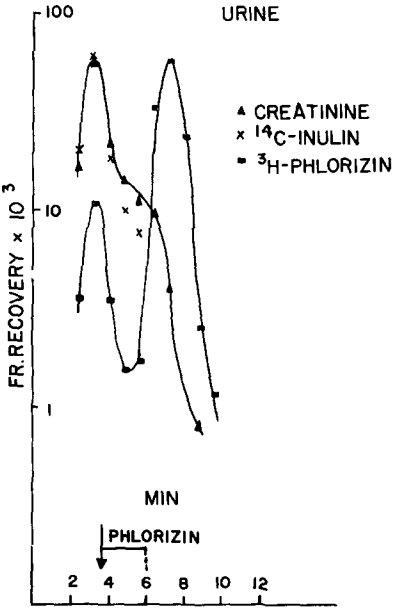

Fig. 8. Urine outflow curves for creatinine (▲), [^{14}C]inulin (×), and [^{3}H]phlorizin (■). The same experimental protocol as obtained in Figs 4, 5 and 7 was repeated. In this case, the sampling interval was lengthened so as to measure the maximal amount of [^{3}H]phlorizin washed off by a systemic bolus of 200 mg of unlabelled phlorizin. The data indicate (i) that inulin and creatinine are glomerular markers and (ii) that there is an impressive "second" urine peak of excreted radiolabel, approximating the quantity of luminal-bound [^{3}H]phlorizin.

Fig. 8 shows the urine outflow patterns for [^{14}C]inulin, creatinine, and [^{3}H]-phlorizin. The experimental protocol was the same used to obtain the results shown in Fig. 4 except that the sampling time was increased from 20 s per sample to 60 s per sample. The increase in sampling time is sufficient to yield complete recovery of [^{3}H]-phlorizin following "wash-off" with a systemic infusion of cold phlorizin. Since phlorizin has no interaction with the antiluminal surface during the time course of our experiments, we conclude that all of the radioactivity recovered in the urine is derived from isotope which was bound to the luminal surface of the nephron. From the observations that show glucose inhibiting the luminal binding of [^{3}H]phlorizin we conclude that the binding of phlorizin takes place at the level of the proximal tubule at sites closely associated with the glucose transport mechanism.

DISCUSSION

The significance of these in vivo studies with [^{3}H]phlorizin is that they demonstrate the existence of high affinity phlorizin receptors at the brush border of the proximal tubule. We can find no evidence that phlorizin has any interaction with the antiluminal surface during the time course of our study (approx. 15 s). Indeed, our data suggest that phlorizin has a limited volume of distribution from the postglomerular circulation compared to the extracellular reference creatinine. These results are consistent with either an exclusion volume or a permeability effect from the postglomerular circulation.

These findings agree with our previous results [1–3] which demonstrated that the glucose-transport system at the antiluminal membrane is about 1000 times less sensitive to phlorizin inhibition than is the transport of glucose at the opposing (brush border) surface. The present data do not contradict the previous studies of Braun et al. [11] which implied a secretory mechanism for phlorizin competitively inhibited by paraaminohippurate. Since the time course of our experiments is so brief, it is possible that we would not detect the existence of a slow secretory transport of phlorizin initiated at the antiluminal surface of the proximal tubule. Our interpretations are consistent with Diedrich's observations on dogs given systemic loading doses of [^{14}C]-phlorizin [12]. By removing the kidneys at specific times, Diedrich was able to calculate the ratio of counts in kidney relative to blood as a function of blood radioactivity. His results show that at very low blood levels, the tissue to blood ratio is very high and then falls sharply. This indicates the presence of high affinity receptors somewhere in kidney tissue. As the activity of phlorizin in the blood rises, there is a slow increase of tissue:blood counts, simulating a first order uptake (i.e. secretory process).

The localization of high affinity phlorizin receptors to the brush border of the proximal tubule gives a rationale to help interpret in vitro [^{3}H]phlorizin binding studies carried out on membrane preparations derived from rat kidney cortex [13–15]. These preparations are admittedly heterogeneous [16]. Along with the excellent correlation between in vitro binding of phlorizin analogues [15] and their in vivo inhibitory potency on glucose transport [17] the results of the present study provide further supportive evidence that the observed in vitro high affinity binding site for [^{3}H]phlorizin is the result of drug binding to brush border fragments. Moreover, these phlorizin binding sites must be closely associated if not identical with the glucose transport receptors imbedded in the matrix of the brush border membrane [18].

The possibility that phlorizin can bind to the inside surface of the antiluminal membrane is not ruled out by our studies. However, Kinne (personal communication) has not observed any high affinity receptors in membrane fractions which have been separated into luminal and antiluminal fragments.

ACKNOWLEDGEMENTS

The technical assistance of Mrs A. Chatzilias and the excellent typing of Mrs S. McGugan is gratefully acknowledged.

This work was supported by the Medical Research Council of Canada MA4590 and the Atkinson Charitable Foundation.

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