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RENAL HANDLING OF PHENOL RED. III. BIDIRECTIONAL TRANSPORT

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SUMMARY

1. The renal excretion of phenol red and other phenolsulphophthalein dyes (bromophenol blue and bromothymol blue) was studied in clearance experiments on anaesthetized rabbits.

2. Net tubular excretion of phenol red reached a maximal value of 8 μmole/min at a plasma concentration of ultrafiltrable dye of about 0·1 mM and was decreased at higher plasma concentrations. Decreases in net tubular excretion at high plasma concentrations were also obtained for bromophenol blue and bromothymol blue suggesting tubular reabsorption in addition to tubular secretion of the dye. Conclusive evidence for reabsorption was provided by administration of probenecid which caused a fall in the excretion of the dyes below that filtered by the glomeruli.

3. Tubular reabsorption of phenol red during probenecid administration appeared to be proportional to the glomerular load and was increased under experimental conditions leading to a decrease of urinary pH. Experiments involving efflux of phenol red from liposomes gave no evidence of a significant role of transmembrane passage by non-ionic diffusion. It is suggested that the pH dependence of the reabsorptive process is the result of preferential reabsorption of the acid as compared to the basic form of the indicator dye across a hydrophilic pathway in the transporting membranes.

4. Clearance ratio of phenol red to that of *p*-aminohippurate at low plasma concentrations was about 0·3. The low degree of extraction of phenol red from renal plasma is attributed both to tubular reabsorption and binding of the dye by plasma proteins.

INTRODUCTION

Tubular excretion of organic anions by the kidney comprises a wide spectrum of compounds, including phenol red, *p*-aminohippurate (PAH) and Diodrast. In early studies, phenol red played a key role in defining the properties of renal tubular secretory processes (for a review of these studies, see Smith 1951; Lotspeich, 1959). However, PAH and, to a lesser extent, Diodrast, have largely replaced the dye in studies on the organic anion transport system. However, there are some notable differences in the excretion pattern of phenol red in relation to that of PAH and

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Diodrast, viz. (1) the clearance of phenol red at low plasma concentrations is about 0·5–0·6 that of Diodrast in man (Smith, Goldring & Chasis, 1938) and dog (Ochwadt &Pitts, 1956), (2) the clearance of phenol red is reduced by a rise in plasma concentration, even at low levels, in contrast to that of PAH and Diodrast which primarily follows renal plasma flow under these conditions (Smith, 1951), and (3) an upper level for tubular excretion of phenol red is reached at a lower plasma concentration than for the two other compounds (ref Smith, 1951). The latter finding suggests a high affinity of phenol red for the transport system, but this is in apparent contradiction to the well documented incomplete extraction of the dye from blood plasma during passage through the kidney, even at very low plasma concentrations. Ochwadt & Pitts (1956), in a study on this problem, found that extraction of phenol red was augmented in the dog kidney after perfusion with a solution containing dextrans instead of plasma proteins. They suggested that binding of phenol red to plasma proteins limits the availability of the dye for excretion under normal conditions. Nonetheless, the clearance of phenol red during infusion with a protein-free solution still remained smaller than the simultaneously measured Diodrast clearance.

In connexion with the incomplete extraction of phenol red, it is of interest that Weiner, Garlid, Romeo & Mudge (1961) have obtained evidence that the excretion of the closely related dye chlorophenol red is reversed from net secretion to net reabsorption when the plasma level of the dye is raised. Chlorophenol red was chosen by Weiner *et al.* in preference to phenol red, since it was anticipated that the former dye, because of a higher lipid solubility, might be more amenable to tubular reabsorption by diffusion of the dye in the non-ionized state across the lipid bilayer of the renal cell membranes (non-ionic diffusion). However, Weiner *et al.* (1961) found that the excretion of dye was unaffected by urinary changes in pH, in contrast to other compounds which presumably are reabsorbed by this mechanism (Weiner, 1973).

In the present study, we have re-investigated the renal handling of phenol red and other phenolsulphophthalein dyes in the rabbit, particularly with respect to the excretion of the dyes at high plasma concentrations. We have used probenecid, which is a powerful inhibitor of tubular secretion of organic anions (Beyer, 1954), to provide conditions for demonstration and examination of tubular reabsorption of dye. The results obtained provide an unequivocal demonstration of a reabsorptive mechanism of phenol red and other phenolsulphophthalein dyes. In the case of phenol red this factor in conjunction with rather strong binding of the dye to plasma proteins appears to be mainly responsible for the incomplete extraction of the dye at low plasma concentrations.

METHODS

Materials

The different phenolsulphophthalein dyes used in this study (phenol red, bromophenol blue and bromothymol blue) were obtained from Merck AG, Darmstadt, Germany. Probenecid and PAH were provided by Alfred Benzon A/S Copenhagen, Denmark and Fluka AG, Buchs, Switzerland, respectively. Radioactive materials (hydroxy [^{14}C]methyl inulin and phenol [^{35}S]sulphonephthalein (phenol red) were bought from Radiochemical Centre, Amersham, Buckinghamshire. Egg lecithin Type IIIE and rabbit serum albumin, were purchased from Sigma chemical Company, St Louis, U.S.A. All reagents used in this study were analytical grade.

Clearance experiments

General procedure. The experiments were performed on male rabbits, weighing 2·5–4 kg. The animals were anaesthetized with sodium pentobarbitone (Nembutal, Abbott) and artificial respiration was given through a tracheal cannula. An external jugular vein and a carotid artery were cannulated with polyethylene tubing for intravenous infusion and arterial blood sampling, respectively. A stock solution containing 0·75 % inulin together with tracer amounts of hydroxy[^{14}C]methyl-inulin and various concentrations of phenolsulphophthalein dyes, dissolved in physiological saline, was infused over a period of 20 min. Following this, fluid prepared by mixing equal volumes of stock solution and physiological saline was infused. The infusion rate was usually 2 ml./min. After infusion for 60 min, urine collections were started from a urethral catheter. Each clearance period had a duration of 10 min, and the bladder was rinsed twice with 5 ml. physiological saline at the end of the period. Blood was drawn from the arterial catheter 2 min before the midpoint of the clearance period to correct for urinary dead space. The following groups of experiments were carried out.

TABLE 1. Scheme for administration of phenol red

	Priming dose (mg)	Sustaining dose (mg/min)
(1) Administration	20	4
(2) Administration	50	11
(3) Administration	100	20
(4) Administration	200	32

Excretion of phenolsulphophthalein dyes at various plasma concentrations. After the first two clearance periods, the plasma concentration was raised by quick injection of a priming dose of the dye to be examined (phenol red, bromophenol blue, bromothymol blue). This was followed by a higher concentration of the dye in the infusion fluid. Ten minutes were allowed to pass for stabilization of the new level of dye in plasma and in this period urine collection was discontinued. Urine and blood samples were then collected for two periods as described above. Usually, the plasma level was raised stepwise in this manner 4 times during an experiment. A typical scheme for administration of phenol red to obtain quickly constant levels of the dye in plasma is presented in Table 1. In some of these experiments, probenecid (50 mg/kg) was quickly administered after the fourth administration period, which was worked out on the basis of preliminary experiments.

Effect of probenecid on phenolsulphophthalein dye excretion. In some experiments, probenecid was administered I.V. at a large dose of 150 mg/kg, which was infused over a period of 120 min, to obtain information on bromophenol blue and bromothymol blue excretion at a maximally inhibitory effect of the drug on tubular secretion of the dyes. During the 60–120 min of infusion, blood and urine samples were collected for four clearance periods (see results Table 2).

The dose dependent effect of probenecid on phenol red excretion was examined in five experiments after infusion of the following amounts of probenecid: 4, 4, 8, 32 and 100 mg/kg. The infusion period for each dose was 10 min and was followed by collection of blood and urine for two clearance periods after each administration.

Experiments on phenol red excretion during infusion of large amounts of probenecid to suppress tubular secretion of dye. In these experiments probenecid was infused in amounts of 150 mg/kg during the initial 60 min infusion before clearance determinations, and administration of the drug was continued at the rate of 0·5 mg/kg.min throughout the remainder of the experiment. The object was to examine tubular reabsorption of phenol red during heavy loading with probenecid to suppress tubular secretion of the dye (see Results). The following groups of experiments were carried out. (1) Examination of phenol red excretion at various plasma levels in a similar way as described above. (2) Effect of urinary pH on phenol red excretion. We experienced difficulties in producing an acid urine in this herbivorous species. Rabbits on the ordinary laboratory diet excreted an alkaline urine (pH 7·6) during the clearance periods. Administration of HCl by gastric tube before the experiments was without effect, and respiration in presence of 5 % CO_2 only resulted in a decrease of urine pH to 7·2. We finally adopted the

method used by Poulsen & Praetorius (1954) according to which the rabbits are pre-treated with a diet of oatmeal and sucrose for 3 days and withdrawal of food 1 day before the experiment. Urine pH was in this way lowered to 6–7 during first clearance periods, and was subsequently raised by infusion of 0·30 M-$NaHCO_3$. (3) Effect of diuretic state on phenol red excretion. In these experiments, phenol red excretion was first examined after infusion of physiological saline, containing phenol red, inulin and probenecid, at the rate of 0·5 ml./min. Then a priming dose of 2·2 g Na_2SO_4 was injected intravenously and infusion was continued with physiological saline, containing phenol red, inulin, probenecid and 12 % Na_2SO_4, at the rate of 2 ml./min. Clearance determinations were begun after a discard period of 10 min at which time diuresis had risen considerably. (4) Effect of various compounds on phenol red excretion. After collection of urine and blood samples for two control periods, *m*-hydroxybenzoate or α-aminoisobutyrate was injected I.V. in amounts of 500 mg/kg. This priming dose was followed by a sustaining dose of these compounds of 5 mg/kg.min. Clearance determinations were begun after a discard period of 10 min.

TABLE 2. The effect of probenecid (150 mg/kg) on the excretion of bromophenol blue and bromothymol blue during infusion of dye and mannitol in rabbit

Dye	Probenecid administration	C_{in} (ml./min)	P_{PSP} (μM)	F_{PSP} (μM)	$\frac{UV/P}{C_{in}}$	$\frac{UV/F}{C_{in}}$
Bromophenol blue	–	16·4	6·23	0·149	0·215	9·1
	+	10·2	12·15	0·522	0·026	0·6
Bromothymol blue	–	19·1	417·3	3·1	0·021	1·20
	+	15·4	578·1	15·6	0·007	0·26

Table shows the mean values of two groups of experiments. In each group three experiments were carried out. C_{in}, P_{PSP} and F_{PSP} represent inulin clearance, total concentration of dye and free concentration of dye in plasma, respectively. $\frac{UV/P}{C_{in}}$ and $\frac{UV/F}{C_{in}}$ represent clearance ratios of total dye clearance/inulin clearance and free dye clearance/inulin clearance, respectively.

Liposome experiments

Liposomes were prepared essentially according to Bangham, Standish & Watkins (1965), and efflux of phenol red was determined by measuring the rate of release of dye trapped within liposomes. The procedure was as follows. Phenol [^{35}S]sulphonephthalein and egg lecithin were added to solutions of 0·01 M-phosphate (pH varying from 5·5 to 8·0 in different experiments) and 0·15 M-NaCl at final concentrations of dye of 0·7 mg/ml. and of phospholipid of 3 mg/ml. The solutions were agitated on a Griffin shaker for 45 min and then ultrasonicated 3 times for 0·5 min at 20 kc/sec on an MSE 100 W Ultrasonic Disintegrator. The resulting preparations were passed through a column of Sephadex G-25 (Coarse grade), 1 × 20 cm, to remove phenol red outside the liposomes. Fractions of each preparation containing the liposomes were pooled and transferred to a cellophane bag and dialysed overnight against 1 l. of an electrolyte solution of the same electrolyte composition as used for the preparation of the liposomes. The following day, the cellophane bags were transferred to large reagent tubes containing 20 ml. electrolyte solution and shaken. The outside electrolyte solution was changed each hour. Efflux of phenol red from liposomes per hour was calculated from the radioactive content of the dialysates relative to the radioactive content of the bags. This calculation is based on the following assumptions. (1) Slow rate of release of phenol red from liposomes. This was the case, since not more than 4 % of the dye was released from liposomes per hour. (2) Equilibration between rate of release of phenol red from liposomes and rate of dialysis. This was ascertained by observing, within counting uncertainty, a constant content of radioactivity in successive dialysates. (3) Effective removal of phenol red outside liposomes. This was confirmed by control experiments in which phenol red was not added until after preparation of the liposomes. Otherwise the samples were handled in the same manner. In these experiments there was no detectable amount of radioactivity in the dialysates.

Reaction of phenolsulphophthalein dyes with rabbit serum albumin. The rate of dissociation of phenol red and bromophenol blue and bromothymol blue from rabbit serum albumin was studied with an Aminco-Morrow stopped flow apparatus. Krebs-Ringer solution containing 0·125 % (v/v) rabbit serum albumin and phenol red 2 mg/100 ml. was mixed with equal volume of Krebs-Ringer solution (pH = 7·4) at 37 °C. Light absorption was registrated by a Beckman DU spectrophotometer, and displayed on a Tektronix Dual Beam oscilloscope.

Analytical methods and calculations. Phenol red, bromophenol blue and bromothymol blue from plasma and urine samples were extracted and analysed in a similar way as previously described (Sheikh, 1972, 1976). *P*-aminohippurate was determined by a diazotation method of Bratton & Marshall (1939), as modified by Smith *et al.* (1945). The concentration of unbound dye was determined in ultrafiltrates of plasma, obtained by centrifugation of a closed cellophane bag in the device previously described (Kragh-Hansen, Møller & Sheikh, 1972). Clearance values are expressed either on the basis of the total concentration of dye in plasma (denoted as UV/P) or in terms of the plasma concentration of ultrafiltrable dye (UV/F), where UV is the excreted amount of dye in urine (μmole/min), P and F the total and ultrafiltrable concentration of the dye in plasma (mM). In most of the experiments, glomerular filtration rate was measured as the clearance of hydroxy[^{14}C]methyl inulin, which was extracted from plasma and urine and analysed as described elsewhere (Sheikh, Møller & Jørgensen, 1972). In few experiments where phenol [^{35}S]sulphonephthalein was employed, the glomerular filtration rate was determined by non-labelled inulin which was analysed as previously reported from our laboratory (Jørgensen, Møller & Sheikh, 1972). In some control experiments, the clearance of probenecid as a function of urinary pH was determined. Probenecid from plasma and urine was extracted and analysed as recently reported by us (Sheikh & Marta Stahl, 1977).

RESULTS

Tubular excretion of phenol red. The results of a representative experiment on the excretion of phenol red at high plasma concentrations of ultrafiltrable dye is shown in Fig. 1. Curve A, which depicts the urinary content of phenol red, is higher than that of dye filtered by the glomeruli (curve B). The net secreted amount of phenol red (curve C = curve A − curve B) rises steeply at low plasma concentrations and attains a maximal value of 8 μmoles/min at around 0·1 mM-free dye in plasma. There appears to be a gradual decline in the net secreted amount of phenol red at higher plasma concentrations. Similar findings were obtained in three other experiments. In all cases, net secretion of dye was diminished at high plasma concentrations, suggesting the existence of a reabsorptive process for phenol red in addition to secretion by the renal tubules. In the experiment shown in Fig. 1, direct evidence for reabsorption of phenol red was provided by administration of probenecid (50 mg intravenously per kg body weight) at the end of the experiment. This resulted in a further decline of excreted phenol red below the level of that filtered by the glomeruli.

Fig. 2 shows the excretion of phenol red at very low concentrations of ultrafiltrable dye in plasma. The clearance of unbound phenol red (UV/F) is decreased from approx. 40 to 8–9 when the concentration of ultrafiltrable phenol red in plasma is raised from 0·16 to 9·9 μM. This reduction of UV/F is solely a consequence of a rise in the ultrafiltered fraction, since the UV/P clearance is approx. 1·3 times that of inulin clearance at both plasma concentrations. Fig. 2 demonstrates that the amount of phenol red excreted by the tubules appears to be proportional to the ultrafiltrable concentration of phenol red in plasma in this concentration range. Furthermore the low UV/F clearance indicates that phenol red is incompletely extracted from renal plasma during passage through the kidney under conditions which may be considered optimal for tubular excretion of the dye.

Fig. 3 compares tubular excretion of phenol red and PAH at intermediate plasma levels. It is seen that the phenol red curve follows a Michaelis-Menten type kinetics whereas PAH secretion is proportional to plasma concentration. The ratio of PAH clearance was 4–5 times that of inulin clearance, indicating that availability of this compound in renal plasma was a limiting factor for the excreted amount in this concentration range. Despite the low UV/P clearance of phenol red (Fig. 2), the dye is excreted more efficiently at low plasma levels than is PAH. The basis for the higher

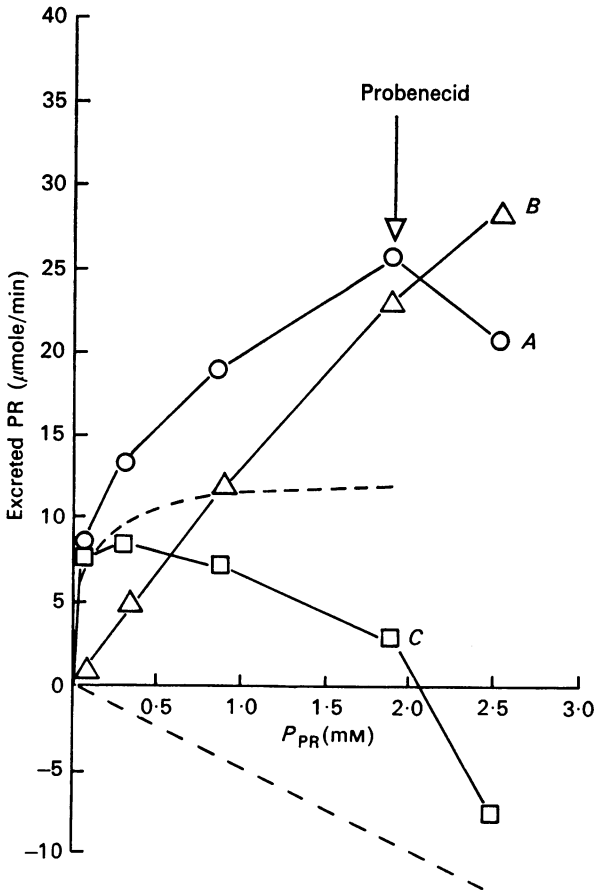

Fig. 1. The tubular titration curve for phenol red (PR) in the rabbit. Curve A indicates urinary excretion of phenol red. Curve B represents the amount of dye filtered by glomeruli. Curve C depicts phenol red secretion calculated as the difference between excretion and filtration ($C = A - B$). The dashed lines indicate hypothetical values for secretory and reabsorptive processes (see text). Ordinate: μmole of phenol red excreted per minute. Abscissa: plasma concentration of free phenol red P_{PR}.

excretion rates of phenol red than of PAH must be provided by the pool of protein-bound phenol red which is released as free phenol red is being secreted by the tubules. However, at high plasma concentrations, secreted PAH is around 60 μmole/min in the rabbit under similar conditions (Møller, 1967) which is around 7–8 times larger than the maximal excretion rate of phenol red (Fig. 1).

Fig. 4 shows that tubular excretion of phenol red is very susceptible to inhibition

by probebecid. Administration of only 4 mg of the compound per kg body weight effected a 73% fall in the UV/F clearance ratio. After administration of 50–150 mg/kg, this ratio was decreased below unity, indicating the existence of a tubular reabsorptive process for phenol red, in agreement with the results of Fig. 1.

Tubular reabsorption of phenol red. It seems reasonable to propose from the above

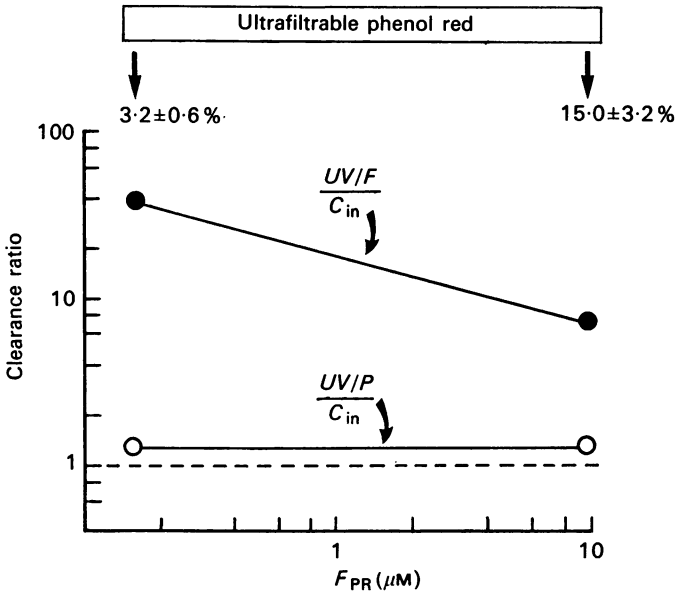

Fig. 2. Excretion of phenol red at very low concentrations of ultrafiltrable dye in plasma. $\frac{UV/F}{C_{in}}$ and $\frac{UV/P}{C_{in}}$ denote the clearance ratios of free and total plasma phenol red/clearance of inulin, respectively. F_{PR} indicates plasma concentration of free phenol red. Note, that radioactive [^{35}S]phenol red is used in these experiments.

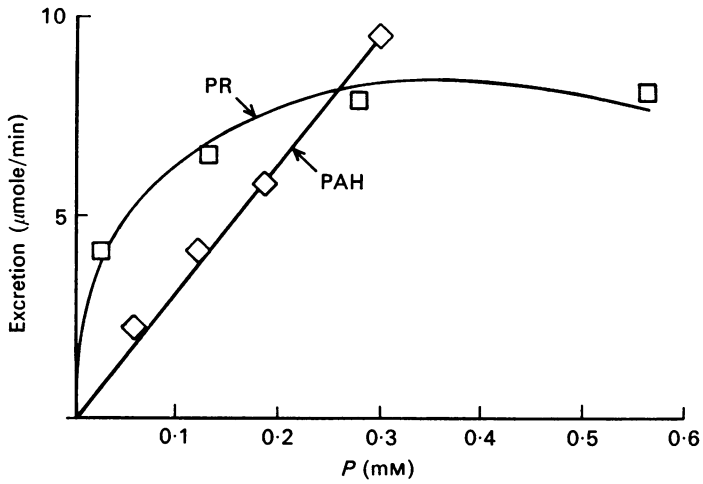

Fig. 3. Comparison between tubular excretion of phenol red (PR) and *p*-aminohippurate (PAH) at intermediate plasma levels. Ordinate: μmole phenol red or PAH excreted per minute. Abscissa: plasma concentration (P) of free phenol red or PAH.

mentioned findings that tubular excretion of phenol red is the resultant of tubular secretion, which approaches saturation level at low plasma concentrations of dye, and tubular reabsorption, which is approximately proportional to plasma concentration of free dye. These proposed characteristics of the secretory and reabsorptive processes are depicted by the interrupted lines on Fig. 1. Evidence for similar excretion curves for salicylate (Weiner *et al.* 1961) and *m*-hydroxybenzoate (May & Weiner,

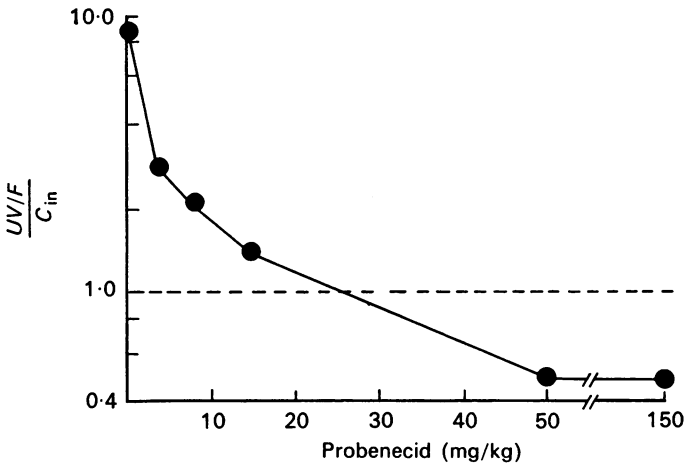

Fig. 4. Effect of probenecid on free phenol red excretion (expressed as clearance ratio). Phenol red (500 μM), mannitol (5 %) and inulin (0·75 %) were infused for 1 hr before clearance determinations. Probenecid was administered in increasing doses of 4, 4, 8, 32 and 100 mg/kg body wt. The infusion period for each dose was 10 min and was followed by collection of blood and urine for clearance determinations.

1970) have been reported, and the approximations involved in these arithmetical representations of tubular secretion and reabsorption are discussed by Weiner (1973). In order to obtain more definitive information on the characteristics of phenol red reabsorption, we have attempted to study excretion of the dye after suppression of tubular secretion by administration of large amounts of probenecid. Fig. 5 shows excretion at different levels of ultrafiltrable phenol red in plasma in an analogous way as Fig. 1. The glomerular filtration rate in these experiments was rather low (10 ml./min), presumably as a consequence of the probenecid administration. Furthermore there is a decline of inulin clearance at the end of the experiment which results in the curved line for glomerularly filtered phenol red (curve *A*). Despite this decline, tubular reabsorption appears to be proportional to ultrafiltrable phenol red (curve *C*). Apparent tubular reabsorption in these experiments constitutes approx. 30 % of the glomerular amount of filtered phenol red.

The excretion of phenol red as a function of urinary pH in oatmeal pre-treated rabbits (see Methods) is shown in Fig. 6. It is seen that the clearance ratio of phenol red and inulin is raised from about 0·15 at pH 5·5 to 0·5–0·6 at pH 8, indicating less reabsorption of the dye by an increase of urinary pH (tubule fluid pH). This finding would be consonant with reabsorption of phenol red by diffusion in the non-ionized state across the cellular membranes, but it should be noted that this interpretation is complicated by the fact that a change in the electrostatic charge of the indicator dye

occurs in this pH region (phenol red is partly converted from the acid (univalent anion) form to the basic (bivalent anion) form with a pK_2 of about 7·8). In order to inquire further into the nature of phenol red permeation through membranes we studied the release of dye from the liposome model system (Methods). The rate constant for efflux of phenol red trapped inside the liposomes was $0·03 \pm 0·006$ hr^{-1} (eight experiments) at 20 °C, independent of changes in medium pH of 5–8. By contrast, probenecid readily leaked out of liposomes during preparation, and the rate

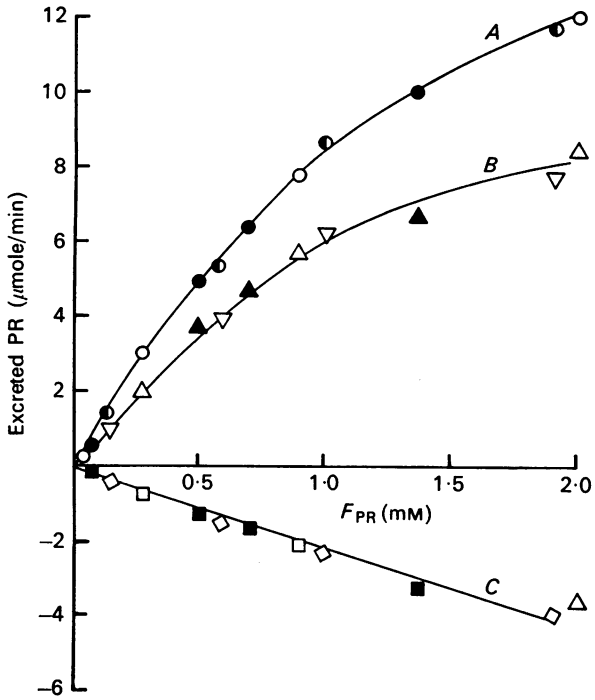

Fig. 5. Excretion of phenol red (PR) at increasing concentration of dye in plasma in the presence of probenecid (see Methods). Curve *A* represents glomerularly filtered phenol red. Curve *B* shows the actual amount of phenol red excreted. Curve *C* indicates the amount of phenol red reabsorbed, calculated as difference between filtration and excretion. F_{PR}, plasma concentration of free phenol red. Different symbols show the results of different experiments.

of release could not be measured with the present methods. The low rate of release of phenol red, which is unaffected by pH, argues against any significant contribution of reabsorption of phenol red by non-ionic diffusion across lipid bilayers. Rather the results suggest that the acid form of the dye is reabsorbed in preference to the basic form through a hydrophilic pathway in the membrane. This would be consonant with the rather large decrease of reabsorption which is observed at a urinary pH of 7·5–8, since pK_2 for the indicator dye is 7·8.

Fig. 6 shows that during a low diuretic level ($V/C_{In} = 0·044$), resulting from the omission of mannitol from the infusion fluid, tubular reabsorption of phenol red was enhanced. Subsequent infusion of Na_2SO_4 was accompanied by a decrease of urinary pH and resulted in the same excretion of phenol red as during mannitol infusion.

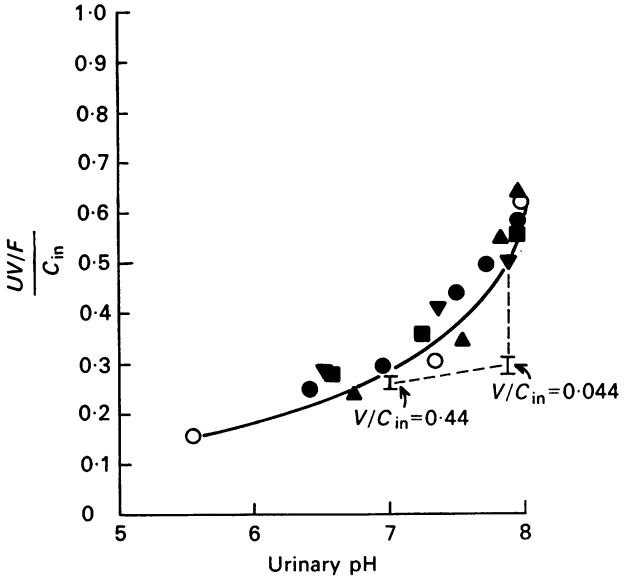

Fig. 6. Excretion of free phenol red (expressed as clearance ratio) as a function of urinary pH. Probenecid was administered throughout the experimental period (see Methods). The dashed lines show the effect of osmotic diuresis on phenol red excretion and urinary pH. Bar denotes standard deviation during 0·15 M-NaCl (V/C_{in} = 0·044) and during infusion of 5% Na_2SO_4 (V/C_{in} = 0·44).

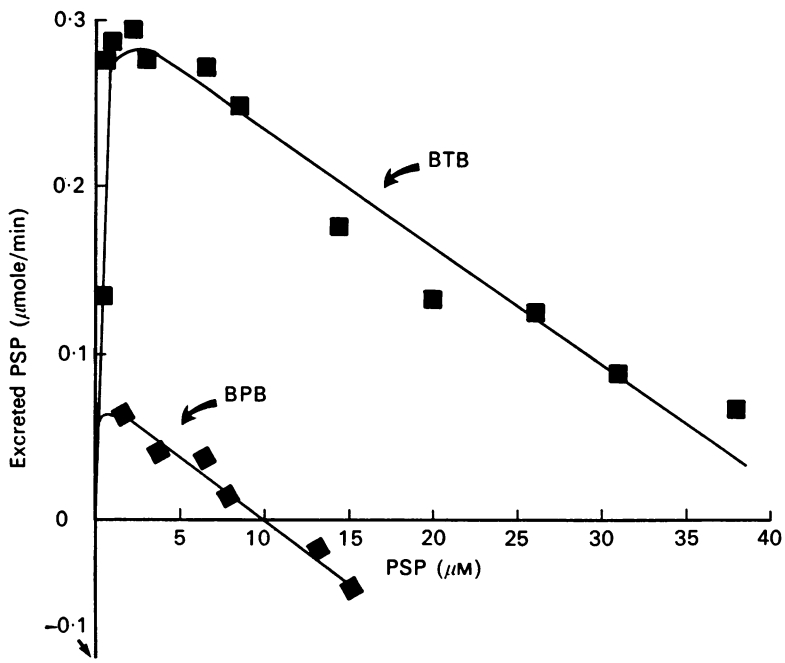

Fig. 7. Excretion of substituted phenolsulphophthalein (PSP) dyes. BPB and BTB denote bromophenol blue and bromothymol blue, respectively. Ordinate: μmole bromophenol blue or bromothymol blue excreted per minute. Abscissa: plasma concentration of free bromophenol blue or bromothymol blue.

Tubular excretion of substituted phenolsulphophthalein dyes. Fig. 7 shows that tubular excretion of bromophenol blue and bromothymol blue as a function of the plasma concentration of free dye exhibits the same features as those of phenol red, i.e. there is a rise of dye excreted by the tubules at low plasma concentrations and a decrease to about the level of glomerular filtration at higher plasma concentrations. The points pertaining to excretion of bromothymol blue should be considered as approximate. Due to a high degree of protein binding ($97{\cdot}3 \pm 0{\cdot}4\,\%$), the exact measurement of the amount of this dye filtered by the glomeruli is difficult to perform. By reference to Fig. 1, it can be seen that maximal secretory rates of bromophenol blue and bromothymol blue are much lower than that of phenol red (maximal amount of tubularly excreted bromophenol blue and bromothymol blue is around 3 and 1 %, respectively, that of phenol red). However, maximal excretion rates are reached at lower plasma concentrations, indicating a higher affinity of the substituted dyes for the transport system, in agreement with previous *in vitro* studies on the uptake of these dyes by isolated flound tubules (Forster & Hong, 1958; Forster, Sperber & Taggart, 1954) and rabbit kidney slices (Sheikh, 1976).

Conclusive evidence of reabsorption of bromophenol blue and bromothymol blue by renal tubules is provided by the results shown in Table 2. As in the case of phenol red, the excretion of the substituted phenolsulphophthalein dyes falls below that filtered by the glomeruli (as evidenced by values of $UV/F \div C_{in}$ below unity) following administration of large amounts of probenecid.

Finally, we have examined the rate of dissociation of phenolsulphophthalein dyes from rabbit serum albumin (see Methods). The results of these experiments reveal that the release of these dyes from the protein is a very quick process. We were unable to measure the dissociation constants of Phenol red and Bromophenol blue with an Aminco-Morrow stopped flow apparatus, since the release of these dyes from rabbit serum albumin occurred within 5 msec.

DISCUSSION

Bidirectional transport of phenolsulphophthalein dyes

The results of the present study demonstrate that various phenolsulphophthalein dyes, in addition to tubular secretion, also are reabsorbed by the renal tubules. Early studies suggested only the existence of tubular secretion of phenol red, since this compound was found to attain a maximal and constant level of tubular excretion at high plasma concentrations (Shannon, 1935; Pitts, 1938). In the present study, the net secreted amount of phenol red was found to approach the level of glomerular filtration at high dye concentrations. Although the reason for this discrepancy with previous studies is not entirely clear, it should be noted that higher plasma concentrations were employed in the present study (Fig. 1) than has generally been the case. Furthermore, by reference to Fig. 1, we see that at high plasma concentrations, the calculated amount of tubular excreted phenol red is subject to some uncertainty because of a relatively high excretion rate of phenol red by glomerular filtration. However, the existence of tubular reabsorption of phenol red is confirmed by administration of probenecid which, owing to inhibition of tubular secretion of phenol red, unmasked the reabsorptive component in the renal handling of the dye. Tubular

excretion curves for bromophenol blue and bromothymol blue were similar to those of phenol red, and for these substituted phenolsulphophthalein dyes, tubular reabsorption was also demonstrated by infusion of probenecid. These observations are in accordance with the findings of Weiner *et al.* (1961) who detected net reabsorption of chlorophenol red at high plasma levels in the dog.

In comparison to phenol red, the affinity and tubular secretion rates of bromophenol blue and bromothymol blue are much lower than those of phenol red. Since most substituted phenolsulphophthalein dyes have been found to be very effective inhibitors of tubular secretion of organic anions (Sperber, 1954; Forster & Copenhaven, 1956; Forster & Hong, 1958), it is probable that the organic anion system binds these dyes more firmly than phenol red and that the transport rate of the substituted phenolsulphophthalein dye is limited by a low turnover number of the transport system. The existence of a small secretory rate of bromothymol blue is in accordance with the results of Sperber (1954) on secretion of phenolsulphophthalein dyes in the chicken, but differs from in-vitro results on the uptake of this dye in rabbit kidney slices where no difference could be detected between the accumulation under anaerobic and aerobic conditions (Despopoulos, 1965; Sheikh, 1976). Although efforts were made in our experiments to optimize conditions for active accumulation of bromothymol blue, it seems probable in retrospect that active accumulation has been obscured by a high degree of passive absorption of the dye to tissue constituents.

Nature of tubular reabsorption of phenol red

We attempted to elucidate the characteristics of the tubular reabsorptive process of phenol red during infusion of large amounts of probenecid to suppress the tubular secretion of the dye. We found that net reabsorption of phenol red under these conditions was proportional to the plasma concentration of unbound dye. This finding constitutes evidence against saturation of the reabsorptive process at tubule fluid concentrations in the millimolar range. Administration of *m*-hydroxybenzoate which shows a similar secretion-reabsorption pattern as that of phenol red (May & Weiner, 1970) apparently did not affect reabsorption of phenol red, nor could any effect be demonstrated by substances like α-aminoisobutyrate and D-glucose which are reabsorbed by the tubule by carrier mediated transport. On the other hand, phenol red reabsorption was very sensitive to changes in urinary pH. This result differs from that obtained by Weiner *et al.* (1961) on the excretion of chlorophenol red in the dog, but is similar to those reported by Lew *et al.* (1962) on the effect of urinary pH on the excretion of other phenolsulphophthalein dyes. The experiments shown in this paper differ from those previously reported in that they were performed during probenecid administration so that any simultaneous effect of urinary pH on the secretory process presumably can be ruled out.

The pH dependence of phenol red reabsorption would be consonant with reabsorption of the dye by non-ionic diffusion across the lipid phase of renal cell membranes, but other observations suggest that caution should be exercised in accepting this conclusion. (1) Measurements of the rate of release of phenol red trapped inside the fluid phase of liposomes showed that diffusion of the dye across lipid bilayer was a slow process, which probably could only account for an insignificant fraction of the amount of phenol red reabsorbed by the kidney. Furthermore, the rate of release of

phenol red from liposomes was independent of pH. (2) Stop-flow experiments suggest that the distal part of the nephron is virtually impermeable to phenol red (unpublished observations in the rabbit). In preliminary, histological localization studies, we have found uptake of phenol red and bromothymol blue to occur in proximal tubule cells with no discernible accumulation in the distal tubules. These results suggest that reabsorption of phenolsulphophthalein dyes is confined to the proximal tubules. If reabsorption of PSP dyes were a process which was governed by non-ionic diffusion across the lipid phase in the plasma membrane, one might have anticipated evidence for reabsorption in the distal part of the renal tubule. Thus experimental evidence for pH dependent reabsorption of salicylate from both proximal and distal part of the nephron was provided in stop-flow experiments by Weiner *et al.* (1959). In conclusion, although phenol red is reabsorbed by a pH dependent process, the behaviour of the dye seems to differ from that of probenecid and salicylate which originally were used to substantiate the theory of non-ionic diffusion. If the reabsorption of phenol red occurs through a hydrophilic pathway in the membrane, as is suggested by the present study, the pH effect is more readily explained by a higher degree of reabsorption of the acid form than of the basic form of the dye, since more reabsorption of the dye was observed by decreasing the urinary pH. The structural basis for a hydrophilic pore, presumably, would be formed by the protein moiety of the plasma membrane, but whether transmembrane passage occurs by interaction with a specific component in the membrane (mediated transport) remains to be elucidated.

Role of protein binding on tubular secretion of phenol red

The clearance of phenol red (UV/P) at low plasma concentrations was 18 ± 3 ml./min while PAH clearance under similar conditions previously was found to be 62 ± 13 ml./min (Møller, 1967). Thus this clearance ratio of phenol red and PAH is 0·3 which is lower than the value of 0·5–0·6 reported for the dog (Ochwadt & Pitts, 1956) and man (Smith *et al.* 1938). This species difference could, at least in part, be due to the high degree of protein binding characteristic of rabbit plasma (Kragh-Hansen *et al.* 1972) as explained below. The question remains to be discussed, how protein binding affects tubular extraction of phenol red from renal plasma. The stop-flow experiments show that dissociation of phenol red from rabbit serum albumin occurs very rapidly and therefore it is reasonable to assume that the release of phenol red from plasma proteins is not the rate limiting step in the secretion of the dye. It appears probable that almost complete extraction of phenol red would occur in the case that the dye were not protein-bound and not reabsorbed by the tubules. This may be inferred from the fact that at low plasma levels, tubular excretion is considerably higher than the amount of ultrafiltrable phenol red presented to the tubules (as evidenced by the high UV/F values at low plasma concentrations, see Fig. 2). Qualitatively, the effect of protein binding on phenol red extraction is suggested in the study of Ochwadt & Pitts (1956) on phenol red excretion in the dog. These authors found an increase of phenol red clearance at low plasma concentrations of dye after replacement of plasma proteins with dextrans. However, the clearance of phenol red was still lower than the simultaneously measured Diodrast clearance (clearance ratio 0·75).

Another important factor in limiting extraction of phenol red from renal plasma

could be tubular reabsorption of phenol red as demonstrated in this paper. Following inhibition of tubular secretion by probenecid, at least approx. 40–50 % of glomerularly filtered dye is reabsorbed. If a similar fraction of tubular secreted phenol red is reabsorbed, this would prevent the extraction of a significant fraction of the dye from renal plasma during passage through the kidney.

The curve of phenol red excretion at relatively low plasma concentrations (Fig. 3), at which there presumably is least interference by tubular reabsorption, resembles that of a carrier-mediated process. In contrast, tubular secretion of PAH in the rabbit under similar conditions is approximately proportional to plasma concentration until plasma levels saturating the transport mechanism is approached (Møller, 1967). This is in agreement with observations on the excretion of these compounds in other species, and has been taken as evidence for lower affinity of phenol red than of PAH for the organic anions transport system (Weiner, 1973). However, it should be considered that the different shapes of the tubular excretion curves may be due to different levels of binding by plasma proteins. PAH is weakly bound (to the extent of 10–15% of total plasma concentration) whereas phenol red binding in the concentration range shown in Fig. 3 varies from 97 to 80%. Protein bound phenol red will act as a reservoir to replenish dye which is excreted by the tubules during passage through the kidney. Since excretion is complete, the concentration of free dye in renal venous plasma will not differ much from that entering the kidney. As a consequence all tubules are presented with a rather uniform concentration of phenol red. In contrast excretion of PAH at low concentration is mainly limited by the amount entering the kidney by renal plasma flow.

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