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EFFECT OF VANADATE ON THE RENAL ACCUMULATION OF *p*-AMINOHIPPURATE IN THE RABBIT KIDNEY TUBULES *IN VITRO*

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Abstract—The possible involvement of Na^+-K^+-ATPase on renal organic anion transport systems was investigated by examining the effect of vanadate and ouabain on the uptake of *p*-aminohippurate (PAH) by rabbit kidney cortical slices. Addition of increasing concentration of vanadate (0.01–0.1 mM) reduced Na^+-K^+ transport and renal steady-state aerobic accumulation of PAH to 80–50 per cent of the control value, without any significant decrease in tissue concentration of ATP. The rapid Na^+-independent component of PAH transport was unaffected by the presence of vanadate (0.1 mM) or ouabain (0.2 mM), whereas the slowly equilibrating component, which makes the largest contribution to the final accumulation of PAH was *ca.* 50 per cent inhibited, by vanadate and almost abolished by ouabain. The anaerobic uptake of PAH was unaffected by a high concentration of vanadate (1 mM), suggesting that vanadate does not competitively inhibit the transport of organic anions. On the basis of the above-mentioned findings, it is suggested that PAH transport is probably linked in a direct manner to the function of Na^+-K^+-ATPase.

INTRODUCTION

There is now general agreement that Na^+ is required to obtain an appreciable accumulation of *p*-aminohippurate (PAH) as a result of active transport by renal tubules *in vitro* [1–3]. In addition the presence of K^+ in the medium is required for optimal accumulation of PAH [4]. Under anaerobic conditions, Podevin *et al.* [2] observed transient accumulation of PAH in the presence of an extracellular to intracellular Na^+-gradient. Since glycolytic processes are not sufficient to drive PAH or Na^+ transport by proximal tubule cells [5, 6], it was concluded that transient accumulation of PAH under anaerobic conditions probably occurs by co-transport with Na^+ [2]. It was further suggested by these authors that active transport of PAH under aerobic conditions is solely dependent on the thermodynamic energy of the Na^+-gradient. However, Gerencser and Hong [1] provided evidence suggesting that accumulation of PAH under aerobic conditions is critically dependent on a sufficiently high intracellular concentration of Na^+, rather than on medium Na^+. They proposed that PAH transport is linked in a direct manner to the function of Na^+-K^+-ATPase. More recently we have concluded that the PAH uptake process can be divided into a rapidly equilibrating component and a slowly equilibrating component, which makes the largest contribution to the final accumulation of PAH [7]. We found that the rapid uptake of PAH is dependent on oxidative metabolism, but is not affected by Na^+. By contrast Na^+ is required for the slow uptake process (for details see Ref. 7).

It has recently been discovered that vanadate is a strong inhibitor of Na^+-K^+-ATPase activity [8, 9]. In the present paper we have examined the effect of vanadate on the uptake process of PAH in rabbit kidney slices. Some supplementary data on the effect of ouabain are also presented. The results obtained emphasize the dependence of the slow component of PAH uptake on Na^+-K^+-ATPase activity.

METHODS

Experimental procedure

Rabbit kidney cortical slices were prepared and incubated in Warburg cups as recently described in detail [7, 10]. The incubation medium contained 10 mM Na^+-acetate, 120–135 mM NaCl, 20–5 mM KCl (to keep the sum of Na^+ and K^+ at 150 mM) 0.5 mM $CaCl_2$, 0.7 mM $MgSO_4$, 15 mM Tris-buffer, (pH = 7.4), (^{3}H)-*p*-aminohippurate (Radiochemical Centre, Amersham, U.K.) and unlabelled *p*-aminohippurate (0.075 mM), to which vanadate or ouabain was added in desired concentrations according to the design of the experiment. There was no difference in the renal uptake pattern of PAH, when Tris-buffer was substituted by CO_2/HCO_3 buffer. The uptake of PAH was measured in a Warburg respirometer under the following conditions: Shaker speed 100 c/min, gas phase 100 per cent O_2 or 100 per cent N_2, temperature 25°, incubation period 1–120 min. The centre well contained KOH for CO_2 absorption. After incubation, the slices were blotted on a piece of filter paper and quickly transferred to conical flasks containing 2.5 ml 5 per cent trichloroacetic acid. The trichloroacetic acid extracts were centrifuged for 10 min at 2000 rpm. A sample of medium (1 ml) was deproteinized by adding 6 ml trichloroacetic acid. Supernatants obtained by centrifugation of the extracts of tissue and media samples were used for the subsequent analyses.

Pretreatment with vanadate or ouabain. In most experiments the freshly prepared slices were exposed to vanadate or ouabain for 30 and 60 min respectively, before measurement of PAH uptake. The

pretreatment took place in an oxygenated medium at 25° containing 10 mM acetate and other electrolytes as required in the subsequent measurement of PAH uptake.

Chemical and radioactive analyses. Radioactive PAH was measured by liquid scintillation counting, using Bray's scintillator [11]. Chemical determination of unlabelled PAH in the supernatants obtained from tissue extracts and media was performed by a diazotation procedure [12] and gave the same value for accumulation of PAH as the radiochemical procedure. The tissue concentration of ATP was determined by enzymatic spectrophotometry as described previously [10]. Concentrations of Na^+ and K^+ in tissue and media were estimated by atomic absorption spectrometry. Inulin space and dry weight were estimated in separate experiments as described by Sheikh and Møller [13].

The accumulation of PAH in the renal tubules was calculated as the difference between the content of this compound in the whole slices and that present in the inulin space. This difference was then divided by the tubular cell water content (final wet wt of tissue minus inulin space minus dry wt) to obtain the tubular cell concentration of the compound. From the latter value the tubular cell accumulation of PAH (T/M_{PAH}) was calculated by division with the medium concentration at the end of the incubation. The tissue concentration of Na^+, K^+, and ATP is given as mmoles/kg final wet tissue wt. Oxygen consumption (Q_{O_2}) is expressed as 1 per kg final wet wt./hr.

RESULTS

Figure 1 shows the effect of a wide range of vanadate concentrations on the steady-state accumulation of PAH, oxygen consumption and the level of ATP, Na^+ and K^+ in rabbit kidney slices incubated in a medium containing 5 mM K^+ and 145 mM Na^+. In order to facilitate comparison between T/M_{PAH} and the various metabolic parameters the results have been plotted as percentage of change from control data obtained in the absence of vanadate. Figure 1 shows that renal accumulation of PAH is decreased to around 80–50 per cent of the control value at 0.01–0.1 mM vanadate, without any effect on the tissue concentration of ATP. A pronounced inhibition of Na^+-K^+ transport as evidenced by a rise of tissue Na^+ and a decrease of tissue K^+ concentrations is observed. A small reduction of renal oxygen consumption was also noted in this concentration range (0.01–0.1 mM). This may be attributed to the decrease of Na^+-K^+ transport which requires a sizeable contribution of the oxygen consumption of the slices [14]. Fig. 1 also shows that PAH accumulation is nearly abolished at 1 mM vanadate, and under these conditions the final tissue concentration of Na^+ is approximately 130 mmol/kg wet wt kidney tissue and remains unchanged despite a further increase of vanadate concentration to 10 mM (not shown). A similar value was previously obtained in the presence of 2 mM CN^-, and it is presumably indicative of complete suppression of Na^+ transport [10]. The tissue concentration of K^+ falls in a reciprocal manner to that of Na^+ and reaches a minimal value at 1 mM vanadate. A modest inhibition of intracellular ATP (80 per cent of control value) together with an increase in oxygen consumption at 1 mM vanadate suggests an "uncoupling" effect on oxidative phosphorylation under conditions of strong inhibition of Na^+-K^+ transport.

It has been shown that vanadate exerts a maximally

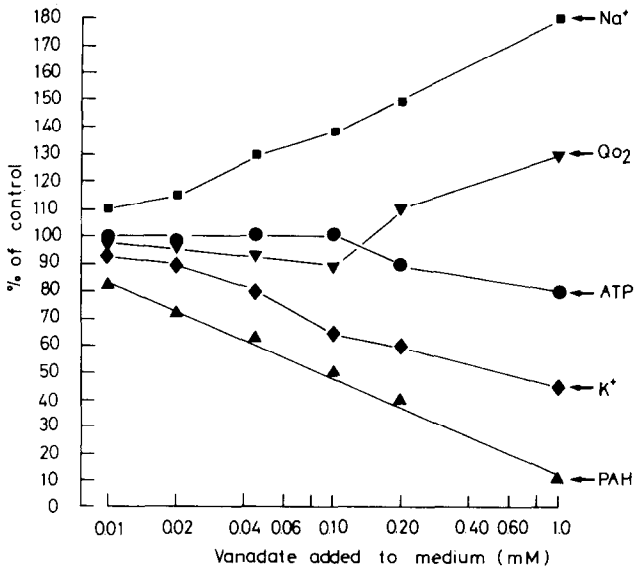

Fig. 1. Influence of vanadate on accumulation of PAH and renal metabolism. The closed symbols indicate aerobic accumulation of PAH (▲—▲), tissue Na^+ (■—■), K^+ (◆—◆), ATP (●—●), and oxygen consumption Q_{O_2} (▼—▼). The initial concentration of PAH used in the experiments described in Fig. 1 and in subsequent figures was 0.075 mM. The control values (in the absence of vanadate) were as follows: T/M_{PAH} 12 ± 1.7, Na^+ 72 ± 1.5 (mmoles/kg wet wt tissue), K^+ 71 ± 3 (mmoles/kg wet wt tissue), ATP 1.95 ± 0.1 (mmoles/kg wet wt tissue), Q_{O_2} 0.82 ± 0.08. The values given in the figure are percentages of those obtained in the absence of vanadate, and indicate the means of duplicate determinations from 4 experiments.

inhibitory effect on Na^+-K^+-ATPase activity of purified renal membranes in the presence of 20 mM K^+ [9]. We noted no statistically significant difference in the inhibition of T/M_{PAH} when incubations were performed at medium concentrations of 20 mM K^+ and 130 mM Na^+, instead of 5 mM K^+ and 145 mM Na^+ as in Fig. 1 (not shown). However, vanadate inhibits Na^+-K^+-ATPase by binding to the intracellular aspect of the protein [15]. Since the intracellular level of K^+ is much higher than in the medium, both at 5 and 20 mM medium K^+, this observation is probably consistent with an intracellular action of vanadate (see Discussion).

Figure 2 shows the time course of PAH uptake in the presence and absence of 0.1 mM vanadate in the medium. In these experiments the slices were preincubated with vanadate for 30 min before addition of PAH in order to equilibrate the renal cells with the inhibitor [16]. Uptake of PAH is characterized by a rapid, Na^+-independent component and a slow, Na^+-dependent component [7]. The inset shows that the rapid component of PAH uptake (i.e. corresponding to the uptake during the first 5 min of incubation) is unchanged in the presence of vanadate, whereas a pronounced decrease in the slow component of PAH uptake is observed. This indicates involvement of Na^+-K^+-ATPase on the Na^+-dependent slow uptake process. In accordance with Fig. 1 the endogeneous level of ATP remained constant during the whole incubation period.

The relation between active PAH accumulation and Na^+-K^+-ATPase was further studied by measurements of the effect of a high concentration (0.2 mM) of ouabain (a specific inhibitor of Na^+-K^+-ATPase) on PAH uptake and Na^+-K^+ concentrations in the kidney slices. Figure 3 shows that the tissue concentration of Na^+ rises continually after exposure to ouabain and reaches a maximal level of 130 mM after 1 hr. The rise in the tissue concentration of Na^+ corresponds to opposite changes in the tissue concentration of K^+ which reaches a minimal value of 26 mmoles/kg after 1 hr. There is no demonstrable effect of ouabain on PAH uptake during the first 20 min, but then the accumulation of the organic anion abruptly declines and reaches the minimal value of 1.5 which is also observed in Na^+-free

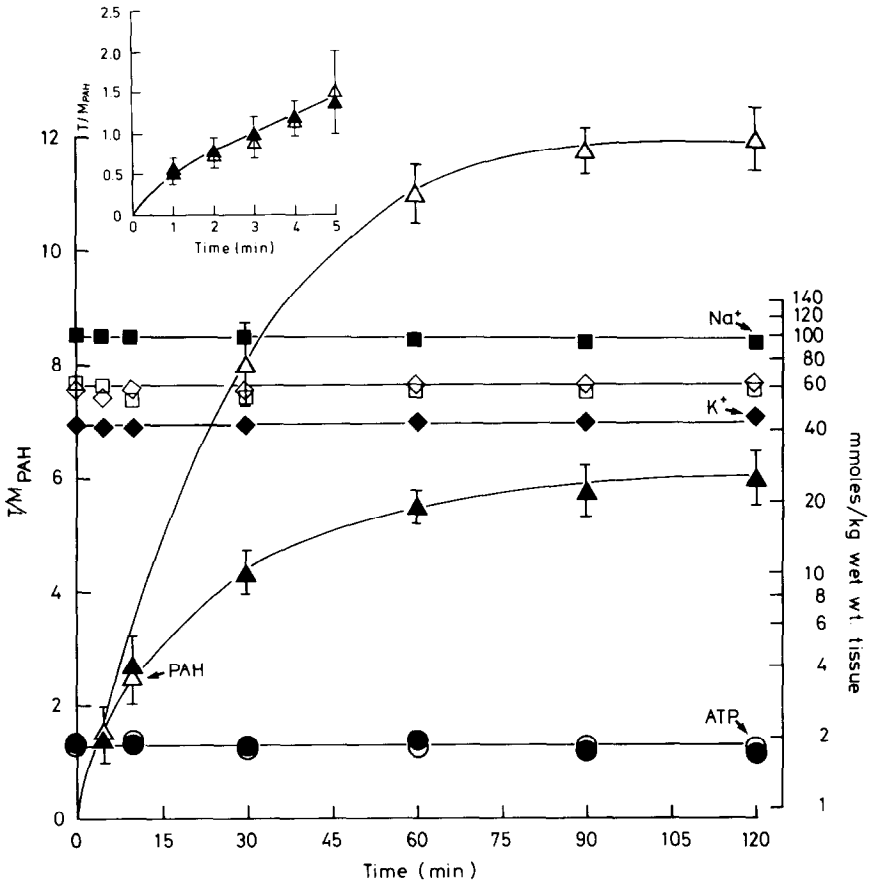

Fig. 2. Effect of 0.1 mM vanadate on aerobic uptake of PAH, tissue Na^+-K^+ and ATP. The inset shows uptake of PAH during initial time of incubation (first 5 min) with and without addition of vanadate. Oxygen consumption (Q_{O_2}) values in these experiments were 0.84 ± 0.05 in the absence of vanadate (control) and 0.75 ± 0.02 in the presence of vanadate. The symbols used are the same as in Fig. 1. Open symbols represent results obtained in the absence of vanadate, whereas closed symbols indicate the effect of vanadate. The values given in the figure indicate the means of duplicate determinations from 5 experiments. Vertical bar denotes S.D.

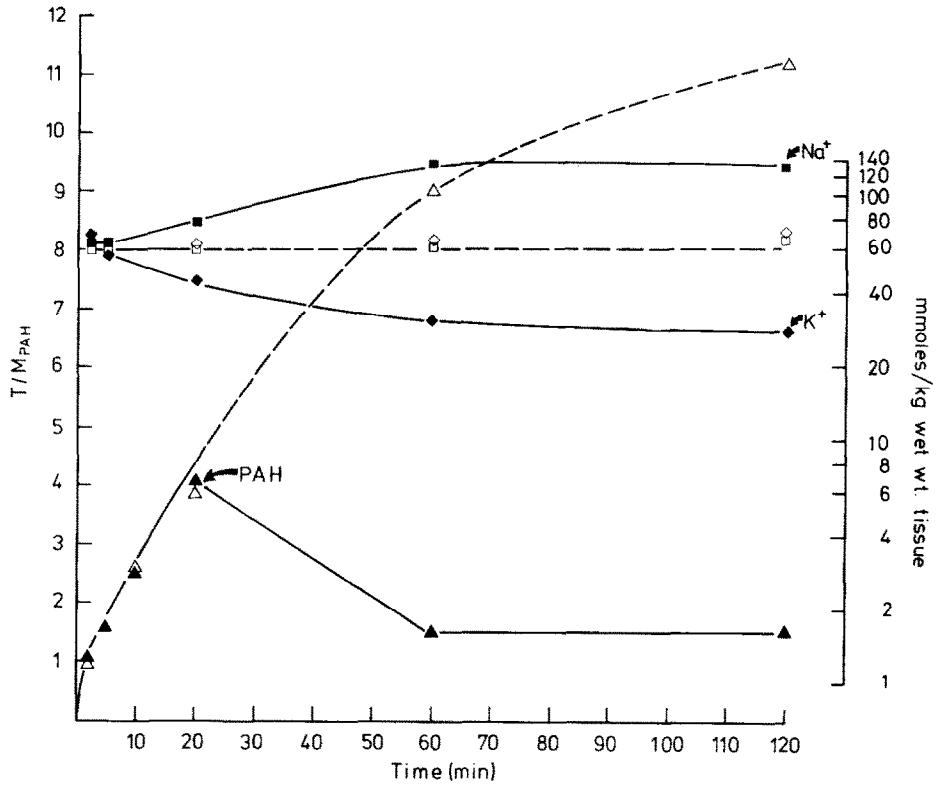

Fig. 3. Time dependent effect of ouabain (200 μM) on uptake of PAH (▲), and tissue concentrations of Na^+ (■) and K^+ (◆). The corresponding open symbols refer to control results obtained in absence of ouabain. The values are the means of 3 experiments.

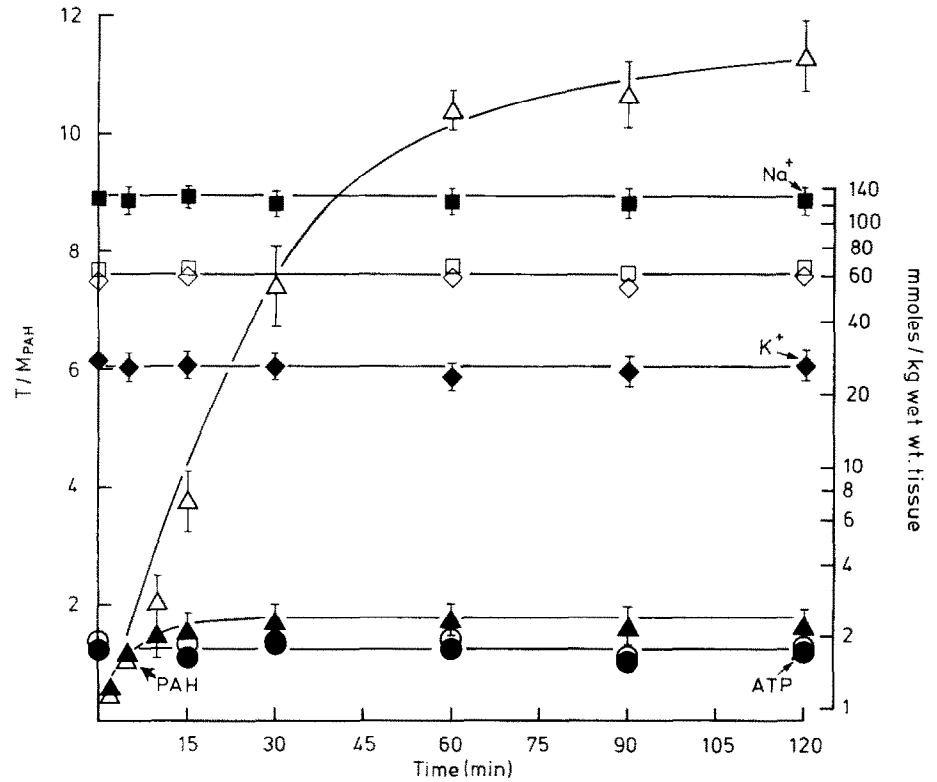

Fig. 4. Effect of 0.2 mM ouabain on aerobic uptake of PAH, tissue Na^+-K^+ and ATP. Oxygen consumption (Q_{O_2}) values in these experiments were 0.83 ± 0.06 in the absence of ouabain (control) and 0.51 ± 0.07 in the presence of ouabain. The symbols used are the same as in Fig. 1. Open symbols represent results obtained in the absence of ouabain, whereas closed symbols indicate the effect of ouabain. The values given in the figure indicate the means of duplicate determinations from 4 experiments. Vertical bar denotes S.D.

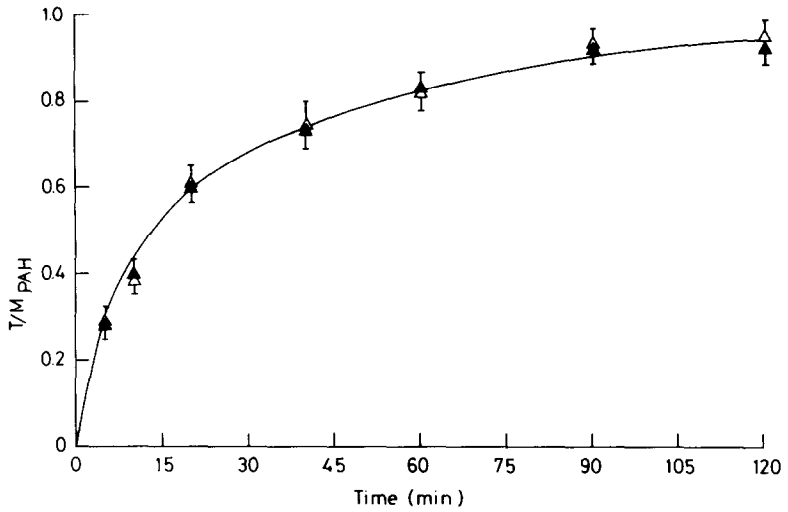

Fig. 5. PAH uptake under anaerobic conditions in the presence of 1 mM vanadate (▲—▲) and in the absence of inhibitor (△—△). The values given are the means of duplicate determinations from 3 experiments. Vertical bar denotes S.D.

incubation media under aerobic conditions (cf. figure 5 of Ref. 10). By contrast PAH uptake continues unabated in the control vessels (no added ouabain) during the first hour. Finally, Fig. 4 indicates that the effects of ouabain is obtained without an effect on the tissue level of ATP.

We also examined the effect of ouabain on PAH uptake 1 hr after addition of the glycoside to the medium. It is seen that the rapid component of PAH uptake is not influenced by the presence of ouabain, while the slow uptake is completely abolished by the glycoside. In accordance with the results of Fig. 3 the intracellular concentration of ATP remains constant during the whole incubation period.

The ouabain data support the results obtained in the presence of vanadate, suggesting involvement of Na^+-K^+-ATPase on slow uptake of PAH in rabbit kidney cortical slices. In order to clarify whether vanadate directly affects PAH transport by inhibition of the carrier function of the transport mechanism, we also studied the effect of this compound on the anaerobic uptake of PAH by renal tissue. It is apparent from Fig. 5 that addition of 1 mM vanadate to the incubation medium does not affect anaerobic T/M_{PAH}, suggesting that this inhibitor does not interact with the PAH carrier system. A similar conclusion was previously reached in the case of ouabain [17].

DISCUSSION

The experimental data reported in this paper confirm and extend our recent observations that indicated the existence of both a Na^+-independent and Na^+-dependent PAH transport mechanism [7, 10, 17]. However, our observations relating to the vanadate results require further consideration. Firstly, medium vanadate concentration which are sufficient to abolish the Na^+-K^+-ATPase activity by *in vitro* preparations [9] only slightly inhibited Na^+-K^+ transport and the steady-state accumulation of PAH in kidney slices. This is probably related to the fact that vanadate inhibits Na^+-K^+ transport by binding to the intracellular aspect of the Na^+-K^+-ATPase [15]. Cantley and Aisen [18] also have reported vanadate to be a much less potent inhibitor of cation transport in whole fat cells than on isolated Na^+-K^+-ATPase preparations. The weak effect of vanadate on Na^+-K^+ transport in intact systems may be accounted for by reduction of the compound from the +5 to the +4 oxidation state (which is only a weak inhibitor of Na^+-K^+-ATPase [19, 20] after cellular uptake. In agreement with this view extensive reduction of vanadate has been shown to take place in erythrocyte preparations [18].

Another question concerning the effect of vanadate relates to the specificity of the compound as an inhibitor of Na^+-K^+-ATPase. A variety of other enzymes has been reported to be inhibited by vanadate [21]. However, among a number of phosphohydrolases the mitochondrial ATPase has been shown to be resistant to vanadate [22, 23]. But our own observations indicate that vanadate does interfere with the energy metabolism of the slices at medium concentrations above 0.1 mM. The decrease in tissue ATP concentration and increase of oxygen consumption is similar to what we have observed previously for 2,4-dinitrophenol [10], suggesting an "uncoupling" effect of vanadate on mitochondrial ATP synthesis.

Since we have no evidence for neither a decrease of renal energy metabolism at 0.1 mM vanadate, nor for a directly inhibitory effect of the compound on PAH (Fig. 5), a causal relationship between the decrease of Na^+-K^+-ATPase activity and PAH accumulation under these conditions is suggested. The ouabain data show that complete inhibition of Na^+-K^+-ATPase activity is accompanied by complete inhibition of Na^+-dependent ouabain PAH transport. Thus the results obtained with the inhibitors of Na^+-K^+-ATPase are in agreement with results obtained in Na^+-free incubation media. They are of

interest, because they indicate that inhibition of PAH transport is directly dependent on the creation of ion gradient caused by the Na^+-K^+-ATPase, and not to some other effect on renal cell function that might be caused by withdrawal of Na^+ from the incubation media. Spencer *et al.* [24] attempted to measure directly the ouabain inhibition of Na^+-K^+-ATPase on homogenates prepared from renal slices. However, they obtained much higher inhibition of Na^+-K^+-ATPase than of PAH accumulation at the same concentration of the glycoside. As noted by the authors this is probably an artefact, because ouabain added to the homogenization and enzymatic assay media probably inactivated additional Na^+-K^+-ATPase molecules. In similar experiments we found that *ca.* 20 per cent of Na^+-K^+-ATPase activity remained in the homogenate at a concentration of ouabain (0.2 mM) which completely inhibited PAH and Na^+-K^+ transport (M. I. Sheikh, J. Maxild and J. V. Møller, unpublished observations). But in contrast to Spencer *et al.* [24] we did not in these experiments add ouabain to the homogenization and enzymatic assay media, so that the possibility of ouabain release cannot be excluded.

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