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The influence of the lipid bilayer phase state on the *p*-aminohippurate (PAH) transport and the activity of the alkaline phosphatase in brush-border membrane vesicles from normal and mutant rats

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The kinetic parameters of *p*-aminohippurate transport and activity of the alkaline phosphatase were studied using brush-border membrane vesicles isolated from the kidney cortex of normal and mutant (strain of Campbell) rats. *p*-Aminohippurate (PAH) transport of both normal and mutant animals was carried out by the mechanism of facilitated diffusion. The apparent Michaelis constant at 36 °C was equal to 7 mM, the maximal rate of PAH transport was 15 nmol/min per mg protein and the constant of inhibition by probenecide was 0.5 mM for normal rats and, respectively, 29 mM, 62 nmol/min per mg protein and 1.4 mM for mutant rats. The Arrhenius plot for the PAH transport and activity of the alkaline phosphatase showed the breakpoints at 28–30 °C for normal rats and at 36–38 °C for the Campbell strain rats. The thermotropic phase transitions detected by the EPR method with 5-doxylstearate as a probe were recorded at 21–30 °C and 30–35 °C for normal and mutant rats, respectively. Therefore, characteristic features of the PAH carrier and alkaline phosphatase activity in normal and Campbell strain rats are determined by the difference in the phase state of their membrane lipid bilayers. We suppose that mutation in the Campbell strain gives rise to a membrane pleotropic effect which enables us to understand the mechanism of genetic control of the lipid structure and membrane fluidity.

Introduction

According to all accounts, the composition and the phase state of lipid bilayer of biomembranes of eukaryotic cells influence substantially the functions of their integral proteins–enzymes [1–6]. The changes of the protein–lipid interactions are often known to be adaptive [6–9]. The character of the protein–lipid interaction must be obviously under genetic control. But there are practically no concrete relevant data which may be due to the absence of suitable experimental models. It would be interesting to compare the action of integral proteins of plasmic membranes (the carrier and the enzyme) of the transporting epithelia cells belonging to animals with normal genotype and those with a mutation leading to changes in the lipid bilayer phase state. The Campbell strain rats, with the autosome recessive gen resulting in the eye disease pigmental retinitis, could be a suitable object for such comparison. This strain has been used for many years as an experimental model to study the disease which is rather common among humans.

It has been recently shown that active transport of organic acids in the epithelium of proximal tubules of the Campbell strain has been broken drastically [10–12]. The temperature dependence of the transport rate of Campbell strain rats differs dramatically from that of normal animals. Besides, the EPR spectroscopy of the spin-labeled basolateral membrane preparations of mutant rats has revealed much higher viscosity of their lipid bilayers [13].

It has been found that in preparations of the Campbell strain rats the fatty acid 20 ($n-3$) is absent and

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the sensitivity of Na^+/K^+-ATPase to ouabain changed substantially. It has been suggested that the mutation of Campbell strain gives rise to alteration of composition and phase state of lipid bilayer of basolateral membrane resulting in modification of their integral proteins action (the carrier and Na^+/K^+-ATPase).

The organic acids transport across the epithelial layer of kidney is known to be carried out by two different carriers localized in the basolateral and apical parts of plasmic membranes, respectively [4,15]. It would be reasonable to suggest that the Campbell strain mutation affects the lipid bilayer phase state not only in basolateral but also in apical membranes. But this influence on the action of organic acid carrier has not been investigated so far even for animals with normal genotype. Therefore, the present research has been aimed at investigating the action of this carrier and of marker enzyme (alkaline phosphatase) as well as the phase state of lipid bilayer in renal-tubule cells of genetically normal and mutant (Campbell strain) rats.

Materials and Methods

The experiments were carried out on white mongrel rats from the nursery Rappolovo (Leningrad district) and the inbred male rats older than two months of the Campbell strain cultivated in the Institute of Evolutionary Physiology and Biochemistry. The animals were killed by decapitation. Their kidneys were removed quickly, decapsulated and placed in the Ringer solution at 0–4°C. The isolation of the apical membrane from the kidney cortex was performed by the cationic precipitation method [16]. The final fraction was resuspended in the medium containing the following ingredients (in mmol/l): mannitol 100, Tris-buffer solution 30, $MgCl_2$ 1 (pH 7.4). The protein concentration in apical membrane fraction was 20–25 mg/ml. In experiments on the organic acid transport across apical membrane *p*-amino[^{3}H]hippuric acid ([^{3}H]PAH) was used with specific activity 3700 MBq/mmol (Amersham) and probenecid (Serva) as a competitive inhibitor. [^{3}H]PAH accumulation in apical membrane vesicles was determined by the rapid filtration method [17] on the nitrocellulose filters (Hemapol, Czechoslovakia) with pore diameter 0.4 μm. Concentrations of [^{3}H]PAH and probenecid varied in different experiments (these data are cited by description of corresponding results). If not specially mentioned, the level of specific PAH transport was determined by the difference between the probes containing [^{3}H]PAH and [^{3}H]PAH plus 5 mmol/l probenecid (probenecid-sensitive transport). The radioactivity was measured on an SL-30 scintillation meter (France).

The inner volume of vesicles was determined as follows. Radioactive inulin with specific activity of 5880 MBq/mmol (Amersham) was added to the freshly obtained apical membrane fraction up to a final specific activity of 1 MBq/ml. Then the sample was passed five times through a hypodermic syringe and incubated during 1 h at 32°C. The same procedure was performed with reference probes but without [^{3}H]inulin. The latter was added to the probe just before filtration. The vesicle specific volume per mg of protein was calculated by the difference in radioactivity between the sample and reference. The purity of preparations obtained was estimated by the activity of the following marker membrane enzymes: alkaline phosphatase (EC 3.1.3.1) after Ebel and others [18], Na^+/K^+-ATPase (EC 3.6.1.3) after Jørgensen [19] and succinate dehydrogenase (EC 1.3.99.1) after Evans [20]. The protein concentration was determined by the method of Lowry et al. [21]. The protein composition of apical membrane was analysed by means of electrophoresis in 10% polyacrylamide gel with sodium dodecylsulfate [22]. The vesicles (5 μg protein) were treated preliminarily by 2% sodium dodecylsulfate. α-Gelatin (95 kDa), bovine serum albumin (68 kDa) and ovalbumin (45 kDa) were used as standards. The gel was stained by 0.1% Coomassie solution G-250. The electron paramagnetic resonance (EPR) spectra of the apical membrane preparations were registered on the nonmodulating spectrometer [23] with 5-doxylstearate (Aldrich Chem. Co.) as a spin probe. The probe was introduced by the method of Hise et al. [24]. The probe/lipid molar ratio did not exceed 1%. The order parameter, S, was calculated by the external and internal extrema of first derivative EPR-spectra with Gaffney correction [25]. The morphology of apical membrane fraction was checked by means of electron microscopy. The pelleted apical membrane vesicles were fixed in 1% glutaraldehyde, dehydrated and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a TESLA-613 electron microscope.

For the experiment the apical membrane fraction was isolated from the kidney cortexes of 10–15 rats, repeating each experiment 3–5 times. Kinetic parameters, apparent Michaelis constant (K_m), maximal rate (V_{max}) and inhibition constant (K_i), were determined from the Lineweaver-Burk graph after graphic subtraction of the diffusion component.

The experimental data are expressed as means ± S.E., and statistical analyses were performed by Student's t-test with $P < 0.05$ as the significance level.

Results

The membrane fraction from the kidney cortexes of normal and mutant rats, according to the electron microscopic control, consists almost entirely of microvilli typical of apical membrane of renal tubules. These microvilli are 0.08–0.10 μm in diameter and 1.0–1.4 μm in length and contain a clearly distinguishable mem-

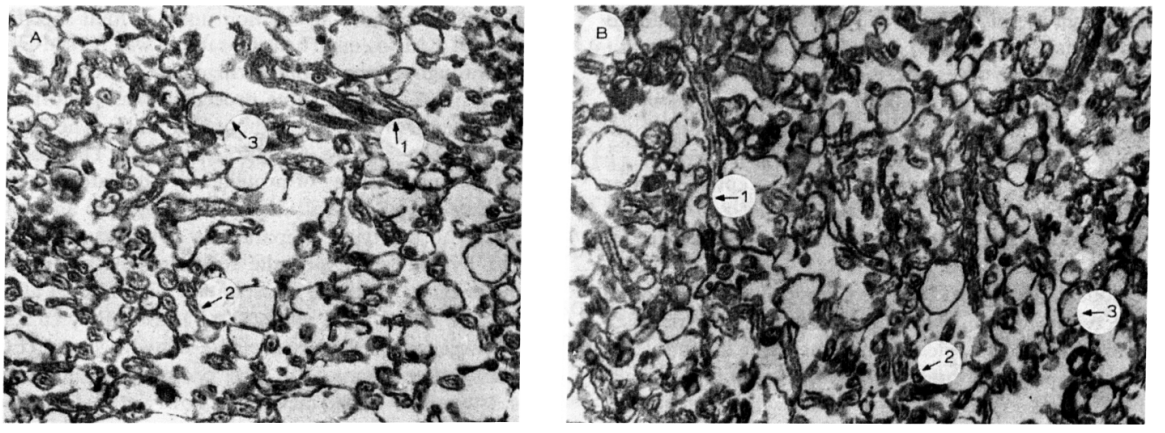

Fig. 1. The micrograph of an ultrathin section of the apical membrane of renal proximal tubules of mongrel (A) and Campbell strain (B) rats. 1, Longitudinal cross-section of microvilli; 2, transversal cross-section; 3, swollen microvilli. Magnification: 31500× (original magnification 42000×).

brane, matrix and central core consisting of microtubules (Fig. 1). There are also some swollen microvilli in the shape of membrane bubbles of 0.3–0.6 μm in diameter, retaining some remains of matrix and central core. Lastly, a small number of rather big (0.5–1.0 μm) closed and opened bubbles are observed; their genesis from microvilli cannot be always proved morphologically. There are no mitochondria, lysosomes and microcorpuscles in the membrane fraction of the rats of both types. An average size of microvillus surface approximated by a cylinder is equal to $27 \cdot 10^{-14}$ cm^2. The internal volume of vesicles-microvilli as measured by equilibration with [^{3}H]inulin was determined to be equal to 0.5 μl/mg protein. Then, the apical membrane vesicle surface is equal to 270 cm^2/mg protein. The data on specific activity of the marker enzymes of apical membrane (alkaline phosphatase), basolateral membrane (Na^+/K^+-ATPase), endoplasmic reticulum membrane (glucose-6-phosphatase) and the membrane of mitochondria (succinate dehydrogenase) in homogenate and in final apical membrane fraction from the kidney cortex of normal and mutant rats are presented in Table I. One can see that the enrichment factors in both cases are practically indistinguishable ($P > 0.05$). The contaminations by other enzymes are negligible. The activity of all enzymes in the homogenate of the Campbell strain and normal rats shows a statistically significant difference. For instance, the activity of alkaline phosphatase of mutant rats is twice as high as that of normal rats.

The electrophoresis of the apical membrane proteins in SDS-polyacrylamide gel testified that the number of bands and their location on the electrophoretograms are identical for both types of animals (Fig. 2). A typical dynamics of probenecid-sensitive [^{3}H]PAH transport in vesicles of apical membrane isolated from kidney cortex

TABLE I

The marker membrane enzyme activity in homogenate and apical membrane fraction of kidney cortex of mongrel and Campbell strain rats

Data presented are means ± S.E.

Animal type	Preparation	Ouabain-sensitive Na^+/K^+-ATPase			Alkaline phosphatase			Glucoso-6-phosphatase			Succinate dehydrogenase		
		S.A. [a]	n [b]	yield (%)	S.A. [a]	n [b]	yield (%)	S.A. [a]	n [b]	yield (%)	S.A. [a]	n [b]	yield (%)
Mongrel	Homogenate	1.4 ±1.3	–	100	4.2 ±2.6	–	100	5.4 ±0.9	–	100	0.3 ±0.1	–	100
	Apical membrane fraction	undetermined	–	–	44 ±5	10.5 ±1.0	13 ±2	1.0 ±0.5	0.20 ±0.05	0.5 ±0.3	undetermined	–	–
Campbell strain	Homogenate	6.2 ±3.1	–	100	10.0 ±2.6	–	100	18.8 ±2.5	–	100	(2.2±0.3) ×10^{-2}	–	100
	Apical membrane fraction	4.7 ±2.6	0.8 ±0.8	1.1 ±0.8	86.0 ±18.0	8.6 ±2.6	11.5 ±5.2	3.5 ±0.5	0.20 ±0.05	0.3 ±0.1	(0.100.05) ×10^{-2}	0.05 ±0.02	0.7 ±0.2

[a] Specific activity (μmol/h per 1 mg protein).
[b] Enrichment factor (relative units).

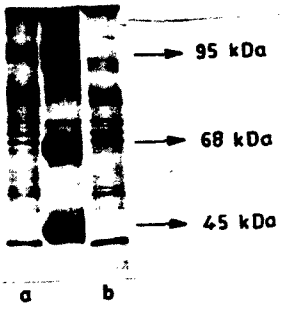

Fig. 2. The electrophoretic separation of proteins of the apical membrane of renal proximal tubules in SDS-polyacrylamide gel; a, Campbell strain rats; b, mongrel rats.

of normal rats at 36°C is presented in Fig. 3 (curve 3). As shown, the transport rate during incubation process decreases and after 60 s becomes equal to zero. In the period between 0 and 30 s the [^{3}H]PAH accumulation level depends linearly on time and therefore the rate within this period is constant and has to be considered as initial. Taking into account the aforesaid intervesicular volume (0.5 μl/mg protein) one can calculate that the PAH concentration in this volume after 60 s is 78 ± 8 μmol/l, i.e., it is practically equal to the PAH concentration in the incubation medium (80 μmol/l). In the case of mutant animals the PAH accumulation dynamics are very similar. The temperature dependence of the initial rate of probenecid-sensitive PAH transport in apical membrane vesicles is bellshaped, the maximum for normal rats occurring at 45–50°C (Q_{10} = 1.3) and for mutant rats at 40–45°C (Q_{10} = 1.8). The Arrhenius plots of these dependences (Fig. 4) show the breaks at 30°C and 37°C, respectively. The dependence of the substrate split rate (*p*-nitrophenyl phosphate) by alkaline phosphatase on the temperature has the same shape with maximum at 50–55°C and Q_{10} equal to 1.2 for both normal and mutant animals. The Arrhenius plot shows clearly in this case breaks at 30°C for normal and at 37°C for the Campbell strain rats (Fig. 5). The dependence of the order parameter, *S*, on inverse absolute temperature for a spin-labeled preparation of apical membrane deviates from linearity in the temperature intervals 21–30°C for normal rats and 28–35°C for mutants (Fig. 6).

Then, the dependence of the initial transport rate of [^{3}H]PAH into apical membrane vesicles from the kidney cortex on its concentration in incubation medium at

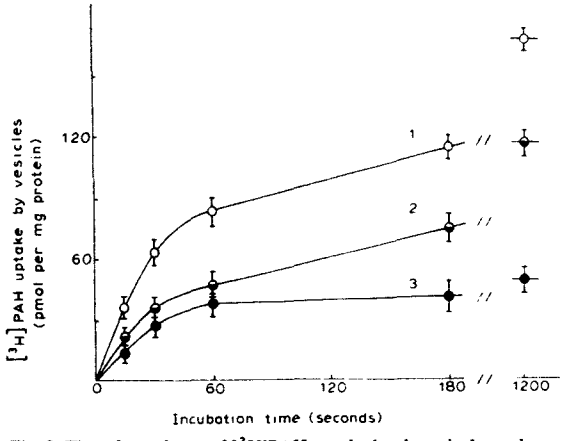

Fig. 3. Time dependence of [^{3}H]PAH uptake by the apical membrane vesicles of mongrel rats at 36°C; 1, without probenecid; 2, in the presence of 5 mM probenecid; 3, differential curve between 1 and 2 (probenecid-sensitive transport); [^{3}H]PAH concentration in medium, 80 μM.

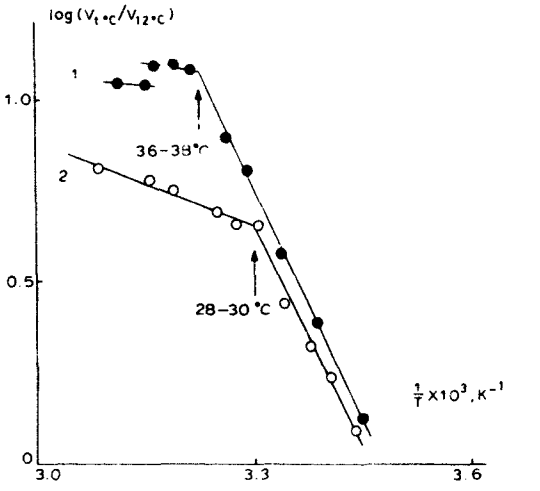

Fig. 4. Temperature dependence of the probenecid-sensitive [^{3}H]PAH transport in the apical membrane vesicles of mongrel (1) and Campbell strain (2) rats. The arrows show the breakpoints.

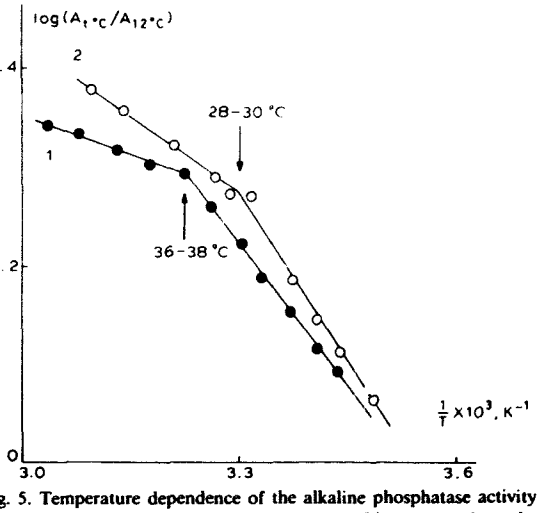

Fig. 5. Temperature dependence of the alkaline phosphatase activity for mongrel (1) and Campbell strain (2) rats. The arrows show the breakpoints.

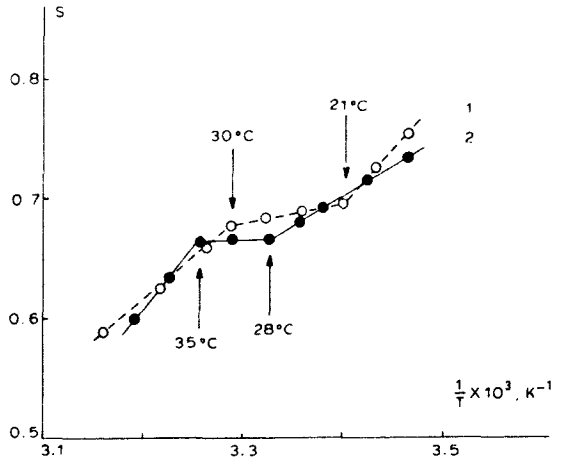

Fig. 6. Temperature dependence of the order parameter S of spin-labeled apical membrane preparations of mongrel (1) and Campbell strain (2) rats. The arrows show the breakpoints.

36°C was studied. One can see (Fig. 7) that the curve reflecting this dependence has saturated and unsaturated components both for normal and mutant rats. In the presence of probenecid in incubation medium the initial rate of PAH transport decreases steadily. After subtraction of the unsaturated component (which is substantially smaller for Campbell mutants) from experimental curves, the transport kinetics in both cases can be satisfactorily described by a Michaelis-Menten equation and its Lineweaver-Burk linear transformation (the correlation coefficient of the experimental points with the straight line exceeds 0.9). It is clearly seen (Fig. 7c, d) that in the presence of probenecid the inhibition of concurrent type takes place. For PAH transport into apical membrane vesicles in the case of normal rats $K_m = 7.0$ mM, $V_{max} = 15$ nmol/min per mg protein, K_i of probenecid = 0.5 mM and those for the Campbell strain rats are: $K_m = 29$ mM, $V_{max} = 62$ nmol/min per mg protein, $K_i = 1.4$ mM. The dependence of the initial rate of substrate (p-nitrophenyl phosphate) splitting on its concentration by alkaline phosphatase at 36°C for both types of rats also satisfies the Michaelis-Menten equation and its Lineweaver-Burk transformation (correlation coefficient is higher than 0.9). For mongrel rats the apparent Michaelis constant of alkaline phosphatase K_m is equal to 0.08 mM and $V_{max} = 59$ μmol/h per mg protein and for the Campbell strain rats, respectively, 0.08 mM and 80 μmol/h per mg protein. Moreover, the initial transport rate of PAH in apical membrane of kidney cortex vesicles on PAH concentration in the medium was examined at 25°C and 22°C. In the first case this dependence in the full interval of the concentrations examined proved to be linear both in the

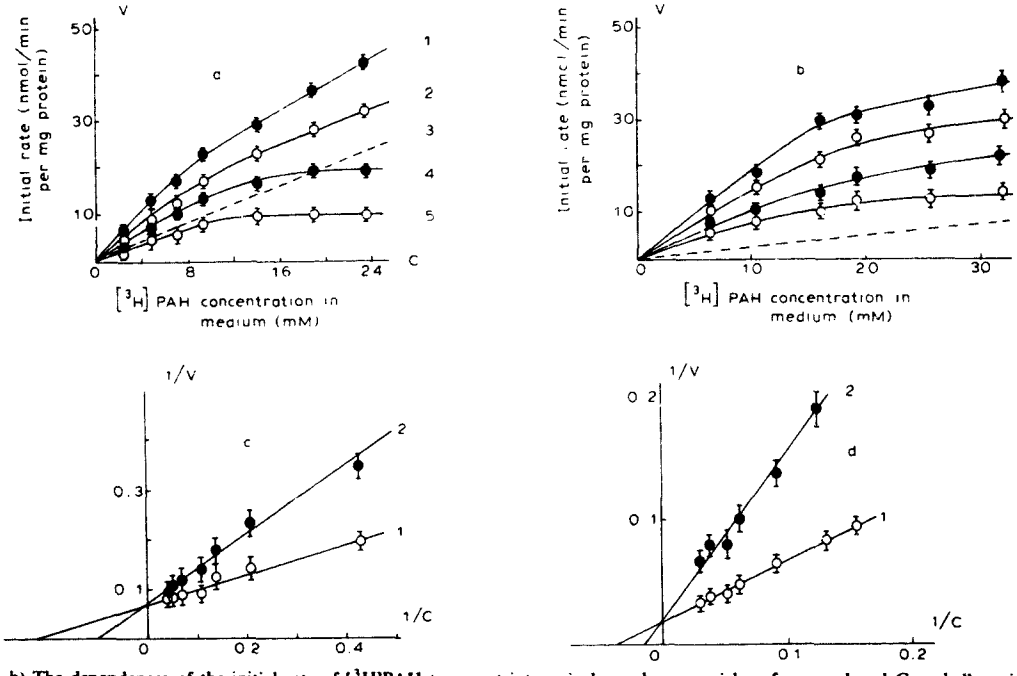

Fig. 7. (a, b) The dependences of the initial rate of [^{3}H]PAH transport into apical membrane vesicles of mongrel and Campbell strain rats on [^{3}H]PAH concentration in incubation medium: 1, without probenecid; 2, in the presence of 0.75 mM probenecid; 3, unsaturated component (straight line, parallel to the linear region of curve 2 corresponding to high concentration of [^{3}H]PAH); 4 and 5, saturated components without (4) and in the presence (5) of probenecid. (c, d) The Lineweaver-Burk plots of the saturated components presented in Figs. 7a and b.

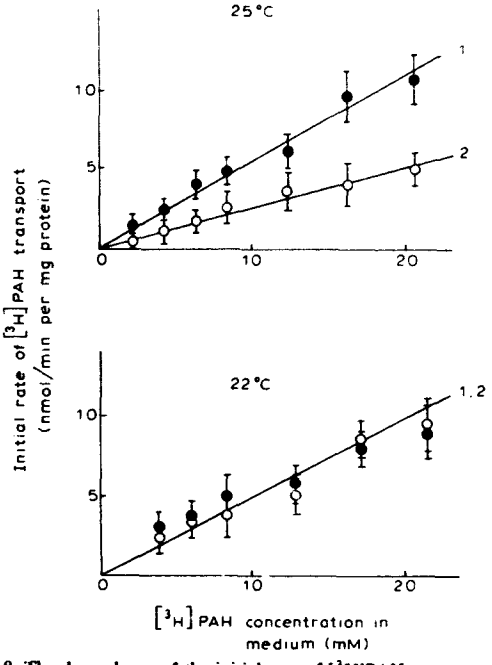

Fig. 8. The dependence of the initial rate of [^{3}H]PAH transport into apical membrane vesicles of mongrel rats on [^{3}H]PAH concentration in medium at 25°C and 22°C; 1, without probenecid; 2, in the presence of 0.75 mM probenecid.

presence and absence of probenecid, though the latter exerts an inhibitory action on transport (Fig. 8a). At 22°C this dependence is also linear but the inhibitory effect of probenecid vanishes (Fig. 8b).

Discussion

The apical membrane fraction of both mongrel and Campbell strain rats consists, as electron microscopic control shows, practically entirely of normal or slightly swollen microvilli of the brush-border tubules. The results of the marker enzyme activity determination in homogenate and final membrane fraction testify that we used a sufficiently pure and homogeneous apical membrane fraction. Our fraction did not differ from that obtained by other investigators in terms of the enrichment factor and the amount of impurities [14,27–31]. Attention should be drawn to the fact that the activity of all marker enzymes investigated in the homogenate differ significantly for normal and mutant rats. Besides, the activity of the apical membrane marker enzyme (alkaline phosphatase) in the final fraction of the Campbell strain rats is twice as high as that of mongrel rats. Probenecid-sensitive PAH transport in apical membrane vesicles continues under our experimental conditions until the PAH concentration inside the vesicles and in the incubation medium becomes equal. So, the motive force of PAH transport in vesicles is its concentration gradient across the membrane. The existence of saturated component, described satisfactorily by the Michaelis-Menten equation, and of concurrent type inhibition of PAH transport by another organic acid (probenecid) leads to the conclusion that PAH transport across the apical membrane is realized by the facilitated diffusion mechanism with special carrier involved. Most other investigators [14,30,32,33] have come to the same conclusion on the basis of their own data. But the PAH carrier kinetic parameters in renal cell apical membrane were determined only in the work of Kinsella et al. [14] made on dog kidney. They were found to be equal: $K_m = (3.87 \pm 0.78)$ mM, $V_{max} = 5$ nmol/min per mg protein, K_i for probenecid = 0.3 mM. Our data on the kinetic parameters of PAH carrier in renal apical membrane of mongrel rats are of the same order of magnitude: $K_m = 7$ mM, $V_{max} = 15$ nmol/min per mg protein, $K_i = 0.5$ mM. But for the Campbell strain rats they differ drastically: $K_m = 29$ mM, $V_{max} = 62$ nmol/min per mg protein, $K_i = 1.4$ mM. Thus the discrepancies in the kinetic parameters of PAH carrier between dogs and rats are much smaller than between normal and mutant rats. It must be admitted that in the apical membranes of normal and mutant rats the V_{max} values of alkaline phosphatase also differ substantially. These differences can be due to the changes of the apical membrane proteins themselves or to the peculiarities of their lipid environment. No differences were found in the electrophoretograms of apical membrane proteins of normal and mutant rats in either the number of bands or in their location. One can conclude therefore that there are no significant modifications in the apical membrane proteins of the Campbell strain rats. That is of course true only for those proteins which can be separated under the electrophoresis conditions used (the molecular mass of proteins is between 30 kDa and 120 kDa). On the other hand, the character of the temperature dependence of organic acid carrier and alkaline phosphatase action in apical membrane indicates the possible involvement of lipid environment. As was shown, there are distinct breaks on the Arrhenius graphs of the initial rates of PAH transport and alkaline phosphatase action temperature dependence (28–30°C for mongrel rats and 36–38°C for the Campbell strain rats). Similar breaks on the Arrhenius graphs of the transmembrane transport rate and the activity of membrane-connected enzyme temperature dependence were recorded by many investigators, who ascribed them to the protein–lipid interaction changes by the phase thermotropic transition [1,4,5, 7–9,13,15,24,29]. This temperature for the preparation of basolateral membranes and that of a endoplasmic net is near 20°C [1,2,9,13,15,34], but in preparation of the apical membrane of renal-tubule and small-intestine cells such breaks were found near 26–29°C and the temperature transition occupied a wide region from

20°C to 30°C [4,5,24,29,35]. The difference in the thermotropic transition temperatures for the basolateral and apical membranes is probably due to the significant differences in their lipid composition. According to our EPR data the region of the thermotropic phase transition in the apical membrane of mongrel rats stretches from 21°C to 30°C and that of the Campbell strain rats from 30°C to 35°C. So, the Arrhenius graph breaks for the PAH transport rates and the substrate splitting by alkaline phosphatase in the preparations of normal and mutant rats are located near the upper limit of the thermotropic phase transition revealed by the EPR method. A dramatic worsening of the affinity between PAH and its carrier (increase of K_m) and the fall of the maximal rate takes place in the apical membrane of normal rats at the temperature corresponding to the middle of the thermotropic transition region. Furthermore, the carrier no longer acts at the temperature near the lower limit of the transition region. Thus, it is these processes developing in the lipid bilayer by thermotropic transition that undoubtedly determine the character of the carrier and the membrane enzyme action. As described earlier in the Introduction, changes were found in the lipid bilayer fluidity of the basolateral membrane of renal-tubule cells and in the properties of the organic acid carrier and Na^+/K^+-ATPase of the Campbell strain rats [10,13]. There are also some alterations in the degree of lipid bilayer microviscosity and the activity of 5'-nucleotidase in the retina membrane of such rats [36–39]. The data presented in Table I show that the activity of Na^+/K^+-ATPase, alkaline phosphatase and succinate dehydrogenase in the kidney cortex homogenates of the mutant and mongrel rats differ drastically, suggesting indirectly that there are alterations in the protein–lipid interaction not only in the apical but also in basolateral membranes, the membranes of endoplasmic net and mitochondria. Therefore mutation leading to the development of pigmental retinitis (a genetic retina degeneration) is accompanied by the pleotropic membrane effects in many, if not all, organs. Such mutations can evidently play an important role both in the development of pathology and in the evolution of eucaryotes and in particular multicellular organisms. Therefore the search for the changes in the cell membrane lipid bilayer phase state by the different mutations of these organisms seems to us to be well worthwhile.

The elucidation of the reasons for changes in the phase state of the apical membrane lipid bilayer of the mutant Campbell strain rats should thus be the goal of further work.

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