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EFFECT OF ACETATE ON TRANSPORT OF ORGANIC ACID (FLUORESCEIN) IN RENAL PROXIMAL TUBULES OF FROG

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The effect of acetate on active fluorescein transport in intact proximal tubules of surviving frog kidney was studied. When the kidneys were incubated in a 120 mM Na^+ medium, 10 mM acetate stimulated fluorescein uptake in the tubules. The stimulation was more pronounced if the kidneys had been previously preincubated for 3 h in the substrate-free solution. Lowering of the Na^+ concentration in the bathing medium to 10 mM resulted in the disappearance of the acetate effect. Preincubation of the kidneys with acetate at 2–4°C gave rise to stimulation of the fluorescein transport only in the 120 mM Na^+ acetate-free medium. The acetate effect on the fluorescein uptake was partially prevented by ouabain. The stimulation of the uptake by acetate in the 120 mM Na^+ medium correlated with an increase in the extent of reduction of pyridine nucleotides in the tubules. The pyridine nucleotides were reduced more markedly after incubation of the kidneys in the 10 mM Na^+ medium, when acetate had no effect on the fluorescein transport. In both the 120 mM and the 10 mM Na^+ media, the cold preincubation of the kidneys with 2.5 mM ADP or 2.5 mM ATP resulted in only slight stimulation of the fluorescein uptake. But in both media the uptake was significantly enhanced after cold preincubation of the kidneys with 2 mM NADH. After the cold preincubation with ADP, stimulation of the fluorescein transport by acetate was observed in the case of the 10 mM Na^+ medium also. The absence of any stimulatory effect of acetate on the organic acid transport in the 10 mM Na^+ medium is explained as the result of the transformation of mitochondria in the tubular cells into the inactive state 4 due to a decrease in the intracellular ADP level. Reducing equivalents are supposed to take part in energization and/or regulation of transport processes in plasma membranes of the renal proximal tubules.

Introduction

Active transport of weak organic acids in renal proximal tubules is known to be dependent on aerobic metabolism [1]. The metabolic substrate, acetate, is a well-known stimulator of the transport system in the tubules [1–10]. One would think that the stimulatory action of acetate involves acetate oxidation in the tricarboxylic acid cycle with a subsequent increase in the intracellular content of ATP generated in mitochondria. However, there are some objections to this suggestion. In the first place, the acetate stimulation of an organic acid (*p*-aminohippurate) uptake in rabbit renal cortex was not accompanied by any augmentation of intracellular ATP level [9]. Secondly, there could be found no close correlation between the rate of *p*-aminohippurate transport in renal tubules and intracellular content of the nucleotide [11,12].

Transport of organic acids in renal proximal tubules is coupled with the active counter-transport (reabsorption) of Na^+ [13–15]. The reabsorption of Na^+ is very likely to possess additional sources of energy, besides ATP [16–18]. The stimulatory

effect of acetate on *p*-aminohippurate uptake in rat renal cortex was not observed if the cortical slices were incubated at low Na^+ concentration [19]. That is why as a starting-point of the present work, a regulatory role of $(Na^+ + K^+)$-ATPase in the mechanism of the stimulatory action of acetate on the organic acid transport system in renal tubules was presupposed. An attempt was made to establish a relationship between organic acid transport and the redox-state of mitochondria in the transporting cells. The results obtained allow us to propose some influence of reducing equivalents on the transport mechanism as well as a regulatory role of $(Na^+ + K^+)$-ATPase in energization of the transport system.

Materials and Methods

Fluorescein (disodium salt, uranin, C.I.45350) was used as a marker organic acid. Fluorescein was shown [20] to share in the frog renal tubules the common transport system with *p*-aminohippurate. The use of a luminescent dye is of advantage because its amount can be determined in intact superficial proximal tubules with the aid of contact microfluorimetry [20].

The present work was carried out on surviving in vitro kidneys of male brown frogs (*Rana temporaria*) weighing 30–50 g. The frogs were kept at 4–8°C before the experiments. The basic physiological solution contained (mM): 117.6 NaCl, 3.4 KCl, 2.4 $NaHCO_3$, 2.8 $CaCl_2$; pH 7.6–7.8 (it will be referred to as the 120 mM Na^+ solution). Additionally, some modifications of the basic solution were used: (1) 10 mM Na^+ solutions—choline and lithium—which contained 110 mM choline chloride or LiCl, respectively, instead of the equimolar amount of NaCl; (2) 120 mM K^+ solution, where all the sodium salts were replaced by appropriate potassium salts; and (3) K^+-free solution, where KCl was replaced by NaCl. The osmolarity and pH of all the solutions were the same. If necessary, the pH was adjusted to 7.8 by HCl or Tris.

The kidneys were incubated in aerated media at 18–20°C, i.e., within the range of the optimal temperatures for the organic acid transport system in frog renal tubules [20]. To load the tubular cells with certain substances or to change the electrolyte composition of the tissue, cold preincubation of the kidneys was used in some experiments. In such instances, pieces (approx. 60 mg) of fresh kidney with intact dorsal surface were immersed in 6–10 ml of an appropriate solution kept at 2–4°C. The preincubation lasted for 2 h without aeration. Before subsequent incubation with fluorescein, the pieces were transferred into the basic solution at 20°C and rinsed for 60 s to raise the temperature of the outer layer on the dorsal renal surface where the proximal tubules under investigation are located. Preincubation of the kidneys in the aerated substrate-free 120 mM Na^+ solution at 20°C for 3 h was also used. Such preincubation was carried out to deplete in part endogenous substrates [19].

The amount of fluorescein accumulated in the intact superficial proximal tubules was measured after incubation of the pieces of kidneys in the bathing medium containing the dye. The measurements were carried out by means of a special microfluorimeter with an objective contact lens. The diameter of the measuring diaphragm in the plane of the object was equal to the average diameter of the proximal tubules (57 μm) [20]. The methods of microfluorimetry and the statistical treatment of data have been described earlier in detail [21]. On the dorsal surface of a kidney the intensities of fluorescein luminescence in 40 proximal tubules were measured. The measurements were repeated on 3–4 frogs, so that each point represents the mean of 120–160 individual records. Fluorescein concentrations in the tubules were calculated with the aid of graduation graph plotted in model experiments with microcuvettes [22]. The results of the transport studies are expressed as the tissue (tubules)/medium ratio (T/M). The data are presented as means ± 2 S.E. The parameters of Michaelis-Menten equation (K_t and V) were calculated from a Lineweaver-Burk transformation by the method of least squares taking into account the statistical weightings [21]. The results were statistically compared using the Student's *t*-test. When the dependence of the fluorescein transport rate on its concentration was investigated, eight concentrations of fluorescein in the bathing medium over the range $2 \cdot 10^{-5}$ to $2.5 \cdot 10^{-4}$ M were used.

To estimate the redox state of tissue pyridine nucleotides, measurements of the blue auto-

luminescence of intact kidney surface were carried out [23]. The maximal oxidation of the pyridine nucleotides was achieved by 0.5 mM 2,4-dinitrophenol, the maximal reduction by 5 mM amytal-sodium. The extent of pyridine nucleotide reduction was evaluated as a percentage $(I_t - I_o) \cdot 100/(I_r - I_o)$, where I_t, I_o and I_r are intensities of blue autoluminescence after incubation of kidneys in test solution, in dinitrophenol solution or in amytal solution, respectively. The intensity of blue autoluminescence (λ_{max} of excitation at 366 nm, λ_{max} of luminescence at 462 nm) was measured with the aid of a special microfluorimeter with a mercury lamp similar that used for measurements of fluorescein luminescence. The method used in this series of experiments is obviously to give an average extent of pyridine nucleotide reduction in the tubular cells as a whole. Intramitochondrial pyridine nucleotides contribute approx. 80% of the total signal from a renal surface [23]. Since amytal and dinitrophenol directly affect the redox state of intramitochondrial pyridine nucleotides only [24], the method described above reveals the alterations of the extent of pyridine nucleotide reduction due to processes occurring in the electron transport chain. The extent of pyridine nucleotide reduction in the surviving renal tissue, as in other tissues [25] has significant individual variability. That is why we compared only the results obtained on pieces of kidneys from the same animal.

The tissue content of Na^+ and K^+ was determined by means of flame photometry after the wet tissue had been digested in 0.1 M HCl. Tissue water was determined gravimetrically from the difference between wet and dried tissue (95°C overnight). Inulin space was used as an estimate of relative volume of extracellular fluid. Inulin was determined by the method of Schreiner [26]. Dry weights of fresh tissue and of tissue incubated for 30 min were $15.9 \pm 0.8\%$ [5] and $18.1 \pm 0.8\%$ [10] of wet weight, respectively. Relative extracellular spaces were $22.0 \pm 6.0\%$ [10] and $14.5 \pm 2.6\%$ [8] after incubations for 90 min in the 120 and 10 mM Na^+ (choline) media, respectively.

Fluorescein was obtained from Koch-Light Laboratories Ltd., ouabain from Calbiochem, Los Angeles, CA. ADP, ATP and NADH (sodium salts) from Reanal, Budapest, Hungary. All other reagents were of commercial grade.

Results

To test the dependence of organic acid transport in frog renal tubules on oxidative phosphorylation, effects of DNP and amytal on fluorescein uptake were studied (Table I). The addition of each of them to the 120 mM Na^+ bathing medium caused a marked inhibition of the uptake. They had small but significant effects on the fluorescein transport in the 10 mM Na^+ medium also. The fluorescein uptake in the inhibitor-free 120 mM Na^+ medium was also inhibited after cold preincubation of the kidneys with these metabolic poisons. It is known (see Ref. 1) that an uncoupler, 2,4-dinitrophenol, can compete with organic acids for a transporting carrier in renal proximal tubules. Therefore, when dinitrophenol is present in the incubating medium (Table I, lines 1 and 2) it can have double inhibitory effect on the fluorescein uptake in the tubules. After cold loading of the tubules by dinitrophenol (Table I, line 3) in its absence from the incubating medium, it could compete with fluorescein only for transfer across the luminal membrane. However, visual control did not show any significant increase in fluorescein content in the cytoplasm. (Competitive inhibition of fluorescein transfer across the luminal membrane did increase intracellular fluorescein content [20].) Intracellular binding of fluorescein is not a rate-limiting step in fluorescein transport in the frog tubules [20]. That is why it is not considered in this context. In the following experiments there is no possibility for dinitrophenol to compete with fluorescein for a carrier in the basolateral membrane.

The capability of acetate in stimulating the fluorescein transport was then investigated. 10 mM acetate slightly but significantly stimulated fluorescein uptake in the tubules of fresh kidneys in the 120 mM Na^+ medium. In the 10 mM Na^+ medium it had no effect (Table II). After preincubation of the tissue for 3 h in the substrate-free solution, the stimulatory effect of acetate on the uptake in the 120 mM Na^+ medium became more pronounced. In the 10 mM Na^+ medium the fluorescein uptake was poorly affected by acetate. Similar data were obtained when Na^+ in the 10 mM Na^+ medium was replaced by Li^+. These results fall into line with data of Evan et al. [19]

TABLE I

INHIBITORY ACTION OF AMYTAL AND DNP ON FLUORESCEIN UPTAKE IN FROG PROXIMAL TUBULES

Cold preincubation (if employed) was for 2 h; fluorescein concentration in bathing medium $5 \cdot 10^{-5}$ M. In the 10 mM Na^+ medium NaCl was replaced by choline chloride.

Conditions		Control	DNP (0.1 mM)		Amytal (5 mM)	
Cold preincubation	Incubation	T/M	T/M	% of inh.	T/M	% of inh.
–	Plus inhibitor, 120 mM Na^+ medium, 30 min	2.66±0.09	1.20±0.04	55	1.19±0.05	55
–	Plus inhibitor, 10 mM Na^+ medium, 15 min	0.80±0.04	0.63±0.02	21	0.64±0.03	20
Plus inhibitor, 120 mM na^+	Minus inhibitor, 120 mM Na^+ medium, 15 min	1.61±0.07	1.05±0.06	35	0.95±0.05	41

who showed that acetate failed to stimulate organic acid transport in rat renal cortical slices at low Na^+ concentration in incubating medium.

It should be noted that in the 120 mM Na^+ medium the stimulatory effect of acetate becomes stronger after preincubation of the tissue for 3 h at 20°C without exogenous substrates. Obviously, the oxidation of some endogenous substrates is the main energy source for the fluorescein transport in proximal tubules of fresh kidneys. That is why an acetate-independent part of the fluorescein uptake does not represent a completely substrate-independent transport. Substrate-independent uptake of fluorescein can be evaluated on the basis of the data obtained at metabolic blockade (for example, Table I, line 3). In this case, T/M for fluorescein uptake is about 1, which coincides with equilibrium T/M for fluorescein uptake in the absence of Na^+ from the bathing medium [21].

It could be suggested that the absence of acetate effect on the fluorescein transport in the 10 mM Na^+ media is due to dependence on Na^+ of acetate penetration into the tubular cells. But this is not the case. In the next series of experiments (Table III) the kidneys before incubation with fluorescein were preincubated for 2 h in various solutions in the cold. As it turned out, cold preincubation of the tissue with acetate resulted in the stimulation of the fluorescein uptake only in the 120 mM Na^+ medium. Cold preincubation in

TABLE II

ACTION OF ACETATE ADDED TO BATHING MEDIUM ON THE FLUORESCEIN UPTAKE

Incubation was for 30 min with $5 \cdot 10^{-5}$ M fluorescein. In the 10 mM Na^+ medium NaCl was replaced by choline chloride.

Preincubation in 120 mM Na^+, 3 h	[Na^+] in incubating medium (mM)	Control T/M	With 10 mM acetate	
			T/M	Effect (%)
–	120	3.87±0.15	4.34±0.07	+12
–	10	2.45±0.08	2.43±0.07	0
+	120	2.64±0.12	4.23±0.18	+60
+	10	1.34±0.05	1.45±0.05	+8

TABLE III

EFFECT OF ACETATE ON FLUORESCEIN UPTAKE IN TUBULES OF KIDNEYS PREINCUBATED IN THE COLD IN VARIOUS SOLUTIONS

Cold preincubations were for 2 h; incubation with fluorescein ($5 \cdot 10^{-5}$ M) was for 30 min. In the 10 mM Na^+ solution NaCl was replaced by choline chloride.

Cold preincubation			Incubation		Fluorescein uptake	
Na^+ (mM)	K^+ (mM)	Acetate (mM)	Na^+ (mM)	Acetate (mM)	T/M	Effect (%)
120	3.4		120		3.91±0.18	
120	3.4		120	10	4.77±0.20	+22
10	3.4		120		3.44±0.14	
10	3.4		120	10	3.96±0.16	+15
10	3.4		10		2.29±0.08	
10	3.4		10	10	2.25±0.08	0
120	3.4		120		3.43±0.11	
120	3.4	10	120		4.10±0.11	+19
10	3.4		10		1.98±0.08	
10	3.4	10	10		1.93±0.07	0
120	0		120		3.32±0.10	
120	0		120	10	3.64±0.12	+10
0	120		120		3.50±0.11	
0	120		120	10	4.72±0.20	+35

both 120 mM Na^+ and 10 mM Na^+ acetate-free solutions per se had practically no effect on either the direction or the extent of the action of acetate on fluorescein uptake during subsequent incubation. It is noteworthy that the acetate effect markedly increased after cold preincubation of the kidneys in 120 mM K^+ solution.

The stimulation by acetate can be detected after quite a short incubation period (5 min). This is true both for tissue preincubated with acetate in the cold and then incubated in acetate-free medium (Fig. 1a) and for fresh tissue in the presence of acetate in the incubating medium (Fig. 1b). With 10 min incubation the dependence of the fluorescein transport rate on the concentration of the dye in the bathing medium with or without acetate (10 mM) was investigated. Acetate was found to stimulate the fluorescein transport at all dye concentrations employed. This stimulation is due to an augmentation of the maximal rate of fluorescein transport: in the control, $K_t = 8.5 \cdot 10^{-5}$ M within 95% confidence limits of from $6.3 \cdot 10^{-5}$ to $13 \cdot 10^{-5}$ M; $V = 28 \pm 5$ μM/min; in the test

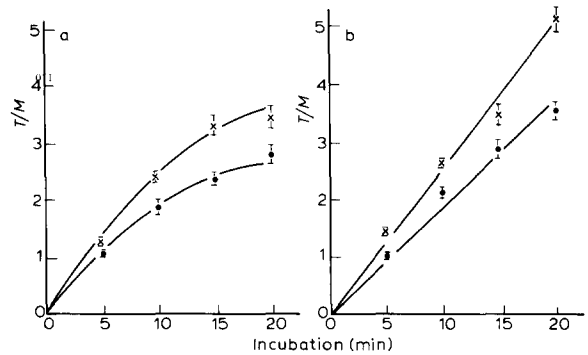

Fig. 1. Time courses of fluorescein uptake in frog renal tubules. Abscissa, duration of incubation (min); ordinate, T/M of fluorescein uptake. (a) The kidneys were preincubated in the cold for 2 h in the 120 mM Na^+ solution with (×———×) or without (●———●) acetate (10 mM) and then incubated at 20°C in the acetate-free 120 mM Na^+ medium. (b) The kidneys were incubated at 20°C in the 120 mM Na^+ medium with (×———×) or without (●———●) acetate (10 mM). Previously they have been preincubated for 3 h in the aerated acetate-free 120 mM Na^+ solution at 20°C. The concentration of fluorescein in the incubating media was $5 \cdot 10^{-5}$ M. The vertical lines show the 95% confidence limits.

(acetate present in the bathing medium), $K_t = 7.8 \cdot 10^{-5}$ M within 95% confidence limits of from $6.5 \cdot 10^{-5}$ to $9.6 \cdot 10^{-5}$ M; $V = 35 \pm 3$ μM/min; the p of the V difference is more than 0.90. Similar data revealing the stimulation of organic acid transport by acetate trhough the augmentation of V have been obtained in studies on renal cortical slices of rat [7] and rabbit [9].

Up to this point there was used only one concentration of acetate (10 mM), the choice being based on data from the literature. The action of lower concentrations of acetate was now investigated (Fig. 2). It turned out that in fresh tissue acetate does not stimulate the fluorescein uptake in the 10 mM Na^+ media (both choline and lithium) and stimulates it very slightly and practically to the same extent in the 120 mM Na^+ medium, at all acetate concentrations employed. In the substrate-depleted tissue the stimulatory effect of acetate is maximal at concentrations of 5 and 10 mM. These data firstly confirm once again the fact of the disappearance of the acetate effect on transport at low external Na^+, and secondly show that 10 mM is a representative concentration of acetate for the study of its effect on the transport.

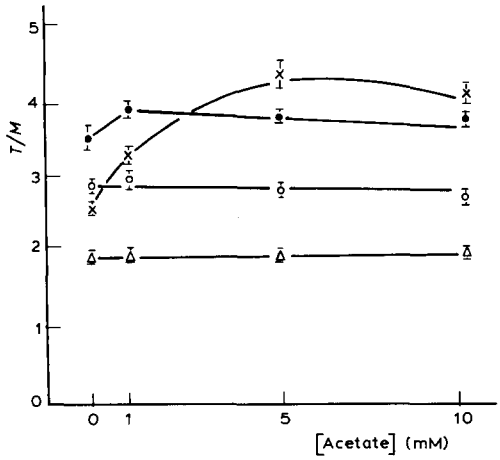

Fig. 2. Dependence of fluorescein uptake on acetate concentration in bath medium. Abscissa, concentration of acetate in bath medium (mM); ordinate, T/M of fluorescein uptake; Fresh tissue: incubation in 120 mM Na^+ medium (●——●), in 10 mM Na^+ (lithium) medium (○——○), or in 10 mM Na^+ (choline) medium (△——△). Kidneys preincubated for 3 h in the aerated substrate-free 120 mM Na^+ solution at 20°C—incubation in the 120 mM Na^+ medium (×——×). All the incubations lasted for 30 min.

In view of the possibility that the absence of any effect of acetate on fluorescein transport in the 10 mM Na^+ media might be related to some alteration in Na^+ transport, we investigated the action of acetate on fluorescein uptake in the presence of ouabain. It is seen (Table IV) that ouabain inhibits the fluorescein transport more sharply when acetate is present in the bathing medium. This phenomenon may be considered as the partial inhibition by ouabain of the acetate effect on the transport.

In a general context it was of interest to investigate the effect of acetate on fluorescein transport after treatment of the tissue by an uncoupler (dinitrophenol) and an inhibitor of the electron transport chain (amytal). The former causes complete oxidation, the latter complete reduction of mitochondrial pyridine nucleotides. It is seen (Table V) that the stimulatory effect of acetate on fluorescein transport is not decreased after preincubation of the tissue with dinitrophenol. After preincubation with amytal the fluorescein transport is even slightly inhibited in the presence of acetate in bathing medium. Note that the effects of the preincubation of the tissue with the inhibitors are somewhat reversible. On the basis of these data it may be assumed that for acetate to stimulate fluorescein transport, the mitochondrial pyridine nucleotides should not be completely reduced.

This assumption is strengthened by the data

TABLE IV

ACTION OF ACETATE ON FLUORESCEIN UPTAKE IN THE PRESENCE OF OUABAIN

The kidneys were preincubated for 3 h in the aerated 120 mM Na^+ substrate-free solution at 20°C; incubation with fluorescein ($5 \cdot 10^{-5}$ M) in the 120 mM Na^+ medium for 30 min; acetate and ouabain were added to incubating medium.

Conditions	T/M	Effect (as compared to the control) (%)
Control	2.03±0.09	
Acetate, 10 mM	2.64±0.11	+30
Ouabain, 0.1 mM	1.71±0.08	−16
Acetate, 10 mM+ ouabain, 0.1 mM	1.97±0.09	0

TABLE V

EFFECTS OF ACETATE ON FLUORESCEIN UPTAKE IN THE TUBULES OF KIDNEYS PREINCUBATED WITH DNP OR AMYTAL

Preincubation of the kidneys with amytal (5 mM) or DNP (0.1 mM) for 15 min in the aerated 120 mM Na^+ solution at 20°C. Incubation with $5 \cdot 10^{-5}$ M fluorescein was for 15 min in the 120 mM Na^+ medium. Acetate was added to incubating medium.

Conditions	Fresh kidneys		Kidneys preincubated with inhibitors	
	T/M	Effect (compared to appropriate control) (%)	T/M T/M	Effect (compared to appropriate control) (%)
2,4-DNP				
Control	3.04±0.12		2.14±0.08	
Acetate, 10 mM	3.29±0.16	+8 [a]	2.55±0.11	+20
Amytal				
Control	2.83±0.12		2.44±0.10	
Acetate, 10 mM	3.33±0.16	+18	2.22±0.08	−9

[a] Effect is significant with $P>0.90$; the other effects, with $P>0.95$.

concerning the extent of reduction of pyridine nucleotides in tubular cells under various conditions of incubation (Table VI). The presence of acetate in the 120 mM Na^+ medium causes some reduction of the pyridine nucleotides. They are reduced more markedly after incubation in the 10 mM Na^+ medium as compared to that in the 120 mM Na^+ medium, especially in the presence of acetate in the media. The more pronounced effects of acetate on the extent of reduction of pyridine nucleotides were observed after preincubation of the tissue for 3 h, i.e., in the case of the substrate-depleted tissue. So, on the one hand, the stimulation of the fluorescein transport in the 120 mM Na^+ medium by acetate correlates with some augmentation of the extent of pyridine nucleotide reduction. On the other hand, absence of acetate effect on the transport is observed when pyridine nucleotides are reduced to a greater extent.

The data of Table VII permit a search for some correlation between the electrolyte composition of the tubular cells and the effects of acetate on the fluorescein transport. It is clear that the development of stimulatory acetate effect on the fluorescein uptake depends on cellular Na^+ concentration precisely in the course of the incubations rather than immediately before them. In fact, after 10 min of incubation in the 120 mM Na^+ medium the intracellular concentration of Na^+ was practically the same in the control kidneys and in the kidneys preincubated in the cold in the 120 mM Na^+, 10 mM Na^+ or K^+-free solutions. Under

TABLE VI

EXTENT OF REDUCTION OF PYRIDINE NUCLEOTIDES IN PROXIMAL TUBULES OF FROG KIDNEYS UNDER VARIOUS CONDITIONS OF INCUBATION

Incubation was for 30 min. Each figure is the arithmetic mean of extent of reduction of pyridine nucleotides obtained on kidneys from six animals. Because of a marked individual variability of results, only the data obtained in the same series may be compared. Differences in paired experiments on pieces of kidney from the same animal were significant with $P>0.80$.

Conditions of incubation		Extent of reduction of pyridine nucleotides (%)	
Na^+ (mM)	Acetate (10 mM)	Fresh kidneys	Kidneys preincubated for 3 h
120	−	31.5	39.5
120	+	37.5	58.5
120	−	34	39
10	−	38.5	46
120	+	42	49.5
10	+	49	62.5

TABLE VII

CONTENT OF Na^+ AND K^+ IN TUBULAR CELLS UNDER VARIOUS CONDITIONS OF INCUBATION

Fresh kidneys after extirpation were rinsed in the 120 mM Na^+ solution for 60 s. During preincubation the kidneys were kept in the cold for 2 h and after being blotted on the filter paper were immersed into 0.1 M HCl. In each experiment pieces of kidney from three animals were used (the principle of paired experiments was applied). Values are mequiv./kg cellular water.

		Before incubation		After 10 min incubation			
				in 120 mM Na^+ medium		in 10 mM Na^+ medium	
		Na^+	K^+	Na^+	K^+	Na^+	K^+
Fresh kidneys (control)		53±6	67±6	52±4	68±4	36±9	56±2
Cold preincubation in solution containing (mequiv./l):							
Na^+	K^+						
120	3.4	52±7	59±6	52±4	64±1		
10 [a]	3.4	37±2	59±5	49±7	57±1		
0	120	32±1	105±5	65±2	82±3		
120	0	64±8	58±1	51±4	64±5		

[a] NaCl was replaced by choline chloride.

these conditions the alterations in fluorescein transport brought about by acetate were very similar, too (Tables II and III). On the other hand, the incubation in the 10 mM Na^+ medium (when the fluorescein transport is not stimulated by acetate) is accompanied by a marked diminution of Na^+ concentration in the cells (Table VII). After 10 min of incubation of the kidneys preincubated in the cold in the 120 mM K^+ solution, the intracellular concentration of K^+ is still higher than in all the other cases. This correlates with the more pronounced stimulation of the fluorescein uptake by acetate after cold preincubation in the 120 mM K^+ solution (Table III). By the way, it may be noted that the cold preincubation in the K^+-free solution does not result in any significant decrease of intracellular K^+ level. Similarly, cold preincubation in the 120 mM Na^+ solutions does not increase Na^+ concentration in the cells. It may well be that these facts are results of the failure of low temperatures to inhibit entirely the activity of $(Na^+ + K^+)$-ATPase in the frog renal tubules. Similar data were earlier obtained with preparations of rabbit kidney [27]. So, it may be assumed that the interference of external Na^+ in the acetate stimulation of the organic acid transport is mediated through dynamic alterations in the intracellular concentration of this cation.

In conclusion, the effects of intermediates of energy metabolism—ADP, ATP and NADH—on fluorescein transport have been studied. ATP and NADH had no effect on the uptake of organic acids in isolated proximal tubules of rabbit when these nucleotides were added to the incubating medium [10]. In the present work cold preincubation of the kidneys with ADP, ATP or NADH was used. In the course of the incubations, the bathing media did not contain organic compounds (except for fluorescein). Such preincubations resulted in significant effects on the fluorescein transport (Table VIII). In the 120 mM Na^+ medium the fluorescein uptake in the tubules preloaded by ADP or ATP was enhanced slightly. When acetate was added to the solutions used for preincubation together with ADP or ATP, their sum effect on the fluorescein transport did not exceed the algebraic sum of the effects of each of them taken separately. Marked enhancement of the transport was observed after cold preincubation of the kidneys with NADH. The stimulatory effect of NADH was also observed in the presence of amytal. In a similar instance, ATP had more pronounced effect

TABLE VIII

FLUORESCEIN UPTAKE IN TUBULES PRELOADED BY ADP, ATP OR NADH

Preincubation of kidneys in the cold for 2 h in the 120 mM Na^+ solution, incubation in the 120 mM Na^+ or 10 mM Na^+ media containing no organic compound except fluorescein ($5 \cdot 10^{-5}$ M) for 30 min at 20°C. In the instance of the 120 mM Na^+ incubating medium, the data of this table were obtained in three series of experiments; that is why there are three different controls. In the instance of the 10 mM Na^+ incubating medium, there is a common control because all the data were obtained in the same series of experiments.

Composition of cold preincubation media (mM)	Incubation in the 120 mM Na^+ medium		Incubation in the 10 mM Na^+ medium	
	T/M	Effect (%)	T/M	Effect (%)
Control I	3.15±0.13		1.17±0.07	
ADP (2.5)	3.41±0.11	+8	1.29±0.05	+10
ATP (2.5)	3.47±0.12	+10	1.31±0.06	+12
NADH (2)	4.56±0.15	+45	1.45±0.06	+32
Control II	2.94±0.11			
Acetate (10)	3.37±0.14	+15	1.21±0.05	0
Acetate (10)+ADP (2.5)	3.53±0.15	+20	1.62±0.05	+38
Acetate (10)+ATP (2.5)	3.54±0.19	+20		
Control III	2.85±0.09			
Amytal (5)	1.88±0.07	−34		
Amytal (5)+NADH (2)	2.38±0.07	−16		
Amytal (5)+ATP (2.5)	2.25±0.08	−21		

(relative) on the fluorescein transport as compared to its effect in the absence of amytal. Results of particular interest were obtained when the after-effects of such preincubations were investigated in the case of the fluorescein transport in 10 mM Na^+ medium. The cold preincubation of the kidneys with ADP, ATP or NADH resulted in effects similar those obtained in the 120 mM Na^+ medium. Fluorescein uptake in the 10 mM Na^+ medium was again unaffected after cold preincubation with acetate. But it was significantly enhanced when the tubules were preloaded with acetate combined with ADP. This implies that the effect of acetate on the fluorescein transport depends on the intracellular level of ADP.

Discussion

Previously we have found that the electrochemical Na^+ gradient on the basolateral membrane of frog renal proximal tubules serves as the principal motive force of the fluorescein transport into the tubules [14,21]. The data from the present work show that the stimulatory effect of acetate on fluorescein transport is revealed only if the Na^+ gradient is directed into the cells. However, the acetate effect on the transport is unlikely to be mediated directly through the Na^+ gradient because the stimulatory action of acetate is due to the augmentation of V, whereas the Na^+ gradient's influence is on K_t [21]. It may hardly be suggested that acetate increases the total amount of carriers for organic acids in plasma membranes of the tubules. It is probable that there are two pools of the carriers: active and non-active. If so, the increase in V for fluorescein transport by acetate could be result of a conversion of non-active carriers into active ones. Also, the Na^+ gradient on the basolateral membrane may affect an affinity to organic acids of the available active carriers. Through V, the fluorescein transport in the frog renal tubules is related to the active counter-flow of Na^+ [14]. Therefore it may be assumed that the acetate effect depends on the activity of ($Na^+ + K^+$)-ATPase.

The regulation of energy metabolism of renal

tissue by $(Na^+ + K^+)$-ATPase is supposed to be due to alterations in the intracellular content of ADP produced during hydrolysis of ATP by the enzyme [28–30]. Additionally, some direct action of Na^+ and K^+ on cellular respiration has been proposed [31] but it is very likely that such effects are artifacts [32].

Our data concerning the absence of the acetate effect on the fluorescein transport in the 10 mM Na^+ medium are to be accounted for by the lowering of $(Na^+ + K^+)$-ATPase activity. In the first place, the stimulatory effect of acetate on the fluorescein transport in the 10 mM Na^+ medium was observed after cold preincubation of the tissue with ADP. Secondly, the acetate effect in the 120 mM Na^+ medium was sensitive to ouabain. Thirdly, the incubation of rabbit renal cortical slices at low Na^+ concentration did in fact result in a significant decrease in the intracellular level of ADP [33]. The data concerning the electrolyte composition of the frog renal tubules are not at variance with the above-mentioned assumption. Really, the decrease of external Na^+ results in the significant lowering of intracellular concentration of this cation (to stimulate $(Na^+ + K^+)$-ATPase just the internal Na^+ is needed). On the other hand, extracellular K^+ is needed at the same time to activate the enzyme. The physiological solution in our experiments contained a rather low K^+ concentration. It is very likely that the main role in activating $(Na^+ + K^+)$-ATPase belongs to K^+ effluxed from the tubular cells into the membrane-side region in close proximity to the basolateral membrane. The rate of K^+ efflux is obviously dependent on its intracellular concentration.

The stimulation of the fluorescein transport by acetate correlates with an increase in reduction of the cellular pyridine nucleotides during acetate oxidation. In the 10 mM Na^+ medium, in which acetate by itself has no effect on the fluorescein transport, the pyridine nucleotides are reduced to a greater extent. In our opinion, this may be accounted for by transformation of part of the mitochondria into the inactive state 4 according Chance and Williams [34]. State 4 is characterized by the complete reduction of mitochondrial pyridine nucleotides due to a decrease in intracellular ADP concentration. It may well be that this is one of the modes of energetic control of substrate oxidation in mitochondria. The control is shown [35] to be accomplished at the level of pyridine nucleotides — flavoproteins of the respiratory chain. The above-mentioned assumption is also supported by the following facts. Ouabain inhibited O_2 consumption by rat renal cortex during the oxidation of a NADH-dependent substrate (β-hydroxybutyrate) but it had no effect on the respiration when succinate or ascorbate (NADH-independent substrates) were oxidized [30]. Additionally, α-ketoglutarate did not stimulate O_2 consumption by rabbit renal cortex in the absence of Na^+ from the bathing medium [28].

Eventual steps of the mechanism by means of which acetate stimulates organic acid transport in the renal tubules are unclear. ATP generated in mitochondria during substrate oxidation may hardly be regarded as the only source of energy used for the active transport (see Introduction). It is very notable that fluorescein transport is stimulated by intracellular NADH in both the 120 mM Na^+ and the 10 mM Na^+ media. It is found [36] that plasma membranes of eucaryotic cells contain NADH-oxidoreductases. Christensen [37] suggested that reducing equivalents produced in mitochondria and transferred into cytoplasm by means of some shuttle system take part in additional energization of transport systems in plasma membranes owing to operation of the oxidoreductases. The results of the present work may be interpreted within the framework of the Christensen's hypothesis. On the other hand, the plasma membrane-bound oxidoreductases interfere in many cellular functions [36]. That is why some regulatory role of reducing equivalents in organic acid transport in renal proximal tubules may not be ruled out.

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