Biochimica et Biophysica Acta, 468 (1977) 81—99

BBA 77719

DOUBLE DEPENDENCE OF ORGANIC ACID ACTIVE TRANSPORT IN PROXIMAL TUBULES OF SURVIVING FROG KIDNEY ON SODIUM IONS

I. INFLUENCE OF SODIUM IONS IN BATH MEDIUM ON THE UPTAKE AND RUN OUT OF FLUORESCEIN AND URANIN

V.M. BRESLER and A.A. NIKIFOROV

Sechenov Institute of Evolutionary Physiology and Biochemistry, Academy of Sciences of U.S.S.R., Leningrad (U.S.S.R)

(Received January 27th, 1977)

Summary

With the aid of direct microfluorimetric determination of marker organic anions (fluorescein and uranin) accumulated in the proximal tubules the influence of Na^+ in the bath medium on the active transport of these anions was studied. Kinetic analysis of the rate dependence of organic acid active transport into tubules on their concentration in the bath medium with a constant Na^+ concentration permitted to define values of apparent K_m and V for uranin and fluorescein transport in the media with different Na^+ content. It was shown that a decrease of Na^+ concentration in the medium increases K_m and lowers the V/K_m ratio with unchanged V. By varying the Na^+ concentration in the medium with a constant concentration of the marker anion the K_{mNa^+} and V_{Na^+} values for fluorescein and uranin transport were determined. A K_{mNa^+} value for fluorescein is twice as much that for uranin. The $1/K_m$ value for uranin transport is a linear function of Na^+ concentration, while for fluorescein transport it is a quadratic one. Therefore it is concluded that two Na^+ from the medium participate in active transfer of one fluorescein anion whereas only one Na^+ from the medium is required for active transfer of one uranin anion. The run out of fluorescein from tubules preloaded with this acid is sharply reinforced by the Na^+ omission from the medium. Thus, active transport of organic acids in proximal tubules of frog kidney is Na^+-dependent, and Na^+ from the medium is likely to participate directly in formation of a transport complex. When Na^+ is absent in the medium a carrier fulfils a facilitated diffusion only.

Introduction

Most investigators suggest that for active transport of a number of organic solutes (sugars, amino acids, organic acids) across plasma membranes of various cells Na^+ from the medium must participate directly in the formation of a transport complex and increase an affinity between a carrier and a substrate (and, consequently, transport rate). One or two Na^+ are supposed to be the constituents of the transport complex, and it moves across the membrane at the expense of energy of Na^+ gradient between the cell and the medium [1—10]. Some authors, however, consider that only intracellular Na^+ influences the organic solutes transport, perhaps, via (Na^+, K^+)-ATPase, while extracellular Na^+ affects the intracellular Na^+ concentration and due to this extracellular Na^+ may influence the organic solutes transport [11—14]. The existence of such contradictions is a result of discrepancies in the experimental data. Therefore it is evident that further improvement of experimental technique for studying the role of Na^+ in the organic solutes transport is called for. In our opinion, the classical system of organic acids active transport in renal proximal tubules would serve an interesting model for such study [15]. There is evidence that the functioning of this system is influenced both by the Na^+ concentration in the medium and by the availability of Na^+ flow from the lumen to the medium across tubular cells [16,18], although facts dealt with action of Na^+ in the medium are rather conflicting.

On the one hand, active transport of organic acids in renal tubules of goldfish [10], lake frog [16] and brown frog [17] was found to be sharply inhibited in the absence of Na^+ in the medium. The kinetic study of transport of organic acid (phenol red) in kidney of goldfish showed an augmentation of an apparent transport K_m when the Na^+ concentration in the medium was being decreased. The $1/K_m$ value was a quadratic function of Na^+ concentration [10]. On the grounds of such kinetics Hoshi and Hayashi [10] came to the conclusion that phenol red is transported by means of formation of a 4-fold complex: carrier plus a substrate plus 2 Na^+.

On the other hand, Gerencser and Hong [19] who worked with cortex slices of the rabbit kidney did not detect extracellular Na^+ influencing either the transport (an affinity between a carrier and a substrate) or the run out of an organic acid from slices. These authors consider that intracellular Na^+ only takes part in the organic acids transport. Thus, it has to be assumed that either cold-blooded animals have a Na^+-dependent system of organic acids transport in kideny whereas in the renal proximal tubules of warm-blooded animals the organic acids transport is not dependent on Na^+, or these differences might be accounted for by some peculiarities of these authors' technique. It should be noted here that in the case of cold-blooded animals the experiments were carried out on a whole and rather intact kidney whereas Gerencser and Hong's work was performed on slices.

So, available facts are contradictory and its checking is called for. It seemed useful to undertake a direct study of the transport process in individual intact proximal tubules. It is shown that it may be done by using a fluorescent organic acid and special contact optics that permits measurement of the concentration of the acid in the tubules of intact kidney through its surface with-

out damaging the tubule structure and with simultaneous visual control [18,20—22]. Preparation of kidney cortex slices leads to the occlusion of tubular lumens with the result that transcellular and transmembrane fluxes of an organic acid become changed as compared to the normal physiological conditions [23]. By using proximal tubules in intact kidney one may avoid such mistakes. The purpose of this work was to study with the aid of the above mentioned method how peritubular Na^+ influences the active transport of two closely related organic anions in the proximal tubules of surviving frog kidney.

Materials and Methods

The experiments were carried out on isolated kidneys of the male frogs *Rana temporaria* collected during the autumn-winter period. The normal salt medium contained (mM/l): 111 NaCl; 3.35 KCl; 2.8 $CaCl_2$; 2.38 $NaHCO_3$; 5.5 glucose; pH 7.4. In the Na^+-free medium Na^+ was substituted by choline and $NaHCO_3$, by $KHCO_3$. When NaCl was substituted by sucrose, 2.38 mM Na^+ remained in the medium owing to $NaHCO_3$. Media with the Na^+ content equal to 3/4; 1/2; 1/4; 1/8 and 1/16 of the normal content (or 85; 56.7; 28,3; 14.2; 7.1 mM/l, respectively), were prepared by substitution of a certain part of NaCl by choline chloride.

In a number of special experiments the substitution of NaCl by LiCl was used. The osmolarity of all the media was the same, pH 7.4—7.5. To control the Na^+ concentration in the lumen and in the capillaries they were washed out by perfusion for 8—12 min with a solution with a required Na^+ concentration. The perfusion was considered to be efficient if the urinary bladder was full and peritubular capillaries contained no erythrocytes. The procedure of perfusion did not influence the transport: after perfusion by a normal medium the average rate of fluorescein transport in the tubules was equal to 13.7 ± 0.7 (working units/min), and in the control kidneys (without perfusion), 14.2 ± 0.7 (working units/min).

As a marker acid fluorescein and its disodium salt (uranin) were used. Ionic and nonionic forms of their molecules in solution and pK values are known [24—26]. The di- and monoanions prevail when pH of the medium is 7.4—7.9. After dissolving fluorescein and uranin in each salt solution used an increase of pH was observed. In weak alkaline solutions of fluorescein a similar phenomenon was noted by Rozwasowski [25]. The final pH of incubation media containing fluorescein or uranin varied from 7.4 to 7.9 as a function of the fluorochromes concentration. Since it was recently shown by the authors of this paper that variations in the pH of the bath medium from 6.5 to 9.0 do not affect very significantly the fluorescein transport in frog proximal tubules [27], the pH of fluorescein and uranin solutions was not adjusted. After perfusion (or in some experimemts, without perfusion) the frog kidney was incubated in a definite salt medium with known concentration of fluorescein or uranin. The temperature of solutions was usually 18—20°C. After incubation the fluorochrome concentration in the tubules was measured by a special microfluorimeter with contact lens [21]. When the effect of Na^+ on the fluorescein run out from tubules was being investigated the kidneys were preincubated for 15 min in the medium with $5 \cdot 10^{-5}$ M fluorescein and then transferred to various

fluorescein-free media. After incubation for 15, 30 or 60 min the concentrations of fluorescein remained in the tubules were measured with the aid of the microfluorimeter. In experiments with a microcuvette it was shown that the intensity of fluorescein luminescence was a linear function of its concentration ranging from 10^{-6} M to 10^{-3} M, i.e. in such fluorescein solutions the concentrative quenching did not occur [28]. The used concentrations of fluorescein (or uranin) in the medium and the time of incubation give in our experiments a maximal fluorochrome concentration in the tubular lumen of about $5 \cdot 10^{-4}$ — 10^{-3} M. Hence, the concentrative quenching of fluorescein (and uranin) luminescence in the tubular lumen may be excluded from consideration. Since in the range of fluorescein (and uranin) concentrations used intensity of tubular fluorochrome luminescence is directly proportional to its concentration, the concentration of fluorescein and uranin in tubules was expressed in working units. They were the units of voltage (mV's) on the output of the photomultiplayer. On each kidney the fluorescence concentrations in 40 proximal tubules were measured and the measurements were repeated on 3—5 frogs. Earlier we described in detail the technique of microfluorimetry [17,20,22]. Constant visual control over the transport was carried out simultaneously with the measurements.

The results obtained by microfluorimetry were computed and the means, standard deviations (σ_i) and confidence limits at the 95% significance level were calculated. The parameters of Michaelis-Menten equation K_m and V were determined from Lineweaver-Burk equation $1/v = K_m/VS + 1/V$ by the method of the least squares [29]. The sequence of determination of K_m, V and their statistical estimations was as follows. The Lineweaver-Burk equation is written as $y - \bar{y} = b(x - \bar{x})$, where $y = 1/v$, $x = 1/S$, $\bar{y}$ and $\bar{x}$ are corresponding mean values, b is a regression coefficient. Our data are transformed to the form: $x_i = 1/S_i$; $y_i = 1/v_i$; statistical weight (ω_i) is equal to $1/\sigma_i^2$, where i = 1,2,3 . . . , n = the number of experimental points. Then

$$\bar{x} = \frac{\sum_i \omega_i x_i}{\sum_i \omega_i}; \quad \bar{y} = \frac{\sum_i \omega_i x_i}{\sum_i \omega_i}; \quad b = \frac{\sum_i \omega_i x_i y_i - \bar{x}\bar{y}\sum_i \omega_i}{\sum_i \omega_i (x_i - \bar{x})^2};$$

$$s_x = \sqrt{\frac{\sum_i \omega_i x_i^2 - \bar{x}^2 \sum_i \omega_i}{(n-1)\sum_i \omega_i}}; \quad s_y = \sqrt{\frac{\sum_i \omega_i y_i^2 - \bar{y}^2 \sum_i \omega_i}{(n-1)\sum_i \omega_i}}$$

The coefficient of correlation with the straight line $\tau = b(Sx/Sy)$ and its estimation

$$\sigma_\tau = \frac{1 - \tau^2}{\sqrt{n}}$$

Estimation for $\bar{y}$:

$$\sigma_y = \frac{Sy\sqrt{(n-1)(1-\tau^2)}}{\sqrt{n(n-2)}}$$

and estimation for b:

$$\sigma_b = \frac{Sy\sqrt{1-r^2}}{Sx\sqrt{n-2}}$$

further $1/K_m = \bar{y}/b - \bar{x}$; $K_m = 1/(\bar{y}/b - \bar{x})$; $V = 1/(\bar{y} - b\bar{x})$, estimation for $1/K_m$ is expressed by the formula

$$\sigma_{1/K_m} = \sqrt{\frac{b^2\sigma_y^2 + y^2\sigma_b^2}{b^4}}$$

[30]. Then the confidence interval for K_m at the 95% significance level is from

$$\frac{1}{1/K_m + 2\sigma_{1/K_m}} \text{ to } \frac{1}{1/K_m - 2\sigma_{1/K_m}}$$

Estimation for V is calculated as

$$\sigma_{V_m} = \sqrt{\frac{\sigma_y^2 + x^2\sigma_b^2}{(\bar{y} - b\bar{x})^4}}$$

and for V/K_m ratio

$$\sigma_{V/K_m} = \frac{\sigma_b}{b^2}.$$

All calculations were made on a programmed computer.

Results

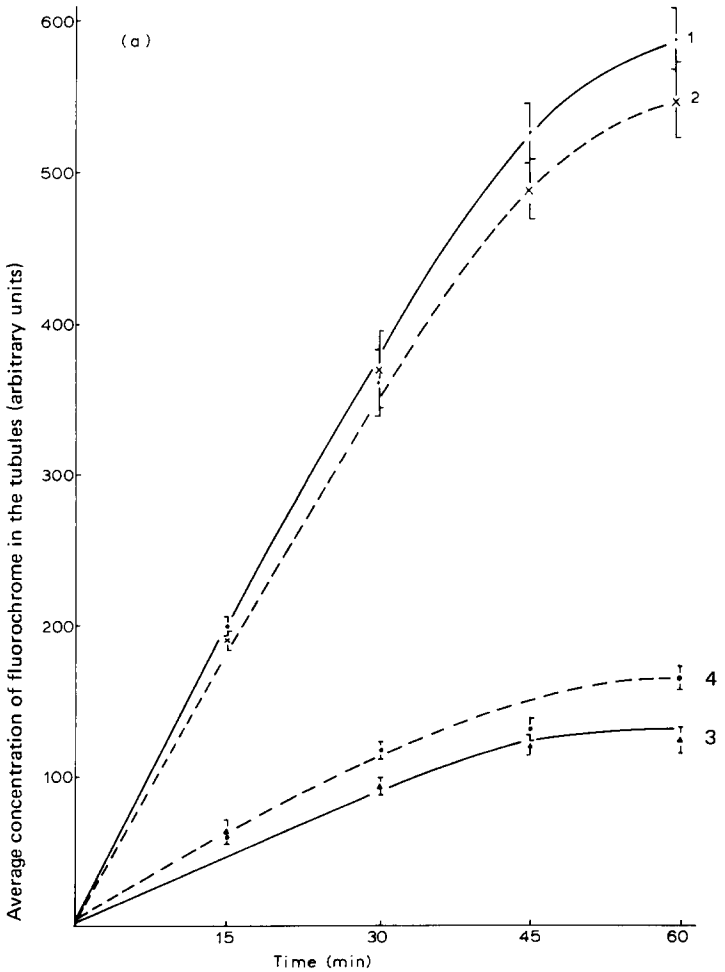
The removal of Na^+ from the tubular lumen and from the bath medium simultaneously inhibits fluorescein and uranin transport by 67 and 65%, respectively. As shown in Fig. 1a the rates of fluorescein and uranin transport are the same in the normal salt medium and until 45 min of incubation the uptake of both fluorescein and uranin depends linearly on the time. It is inhibited in sucrose medium, and as one can see the uptake of fluorescein is inhibited more sharply than that of uranin. This difference is significant. The identical results (although with a less difference) are obtained for the choline chloride medium (Fig. 1b). When Na^+ in the medium is substituted by Li^+ the inhibition of fluorescein transport and breakdown of linear realtionship between the amount of accumulated substrate and the time of incubation are observed. But this inhibition is not so vividly expressed as compared to the Na^+-free media with sucrose or choline chloride (Fig. 1b). Visual control shows that after 45—60 min of incubation in Na^+-free medium the fluorescein concentration in the medium and in the cells becomes equilibrated, whereas uranin concentration in the cytoplasm and in the lumen of tubules in particular is higher than that in the medium.

The increase of fluorescein or uranin concentration in Na^+-free medium (choline chloride) leads to saturation of the transport rate (Fig. 2). This fact offers the possibility to describe these curves with the aid of Michaelis-Menten equation. Indeed, Lineweaver-Burk plots for these data correlate well with the straight line ($r = 0.990$ for fluorescein and $r = 0.985$ for uranin) From

Lineweaver-Burk plots the apparent K_m and V for uranin and fluorescein transport in the normal and Na^+-free media were determined (Table I). In addition, the apparent K_m and V were computed for the net Na^+-dependent transport. For this purpose the uptake rates of fluorescein and uranin in the Na^+-free medium were subtracted from the corresponding uptake rates of these substances in the normal medium (Table I).

When NaCl is substituted by LiCl saturation of fluorescein transport rate occurs as well. In the case of a medium with Li^+ instead of Na^+ the apparent K_m for fluorescein transport does not differ significantly from that for the choline chloride medium, whereas V for Li^+-medium is significantly higher than that for the medium with choline chloride (Table I).

Besides fluorescein, *p*-aminohippuric acid was added to the Na^+-free (choline chloride) medium with the ratio of concentrations *p*-aminohippurate/fluorescein equal to 10 or 80. This addition led to an inhibition of fluorescein uptake in proximal tubules after 30 min of incubation by 22 and 64%, respectively.



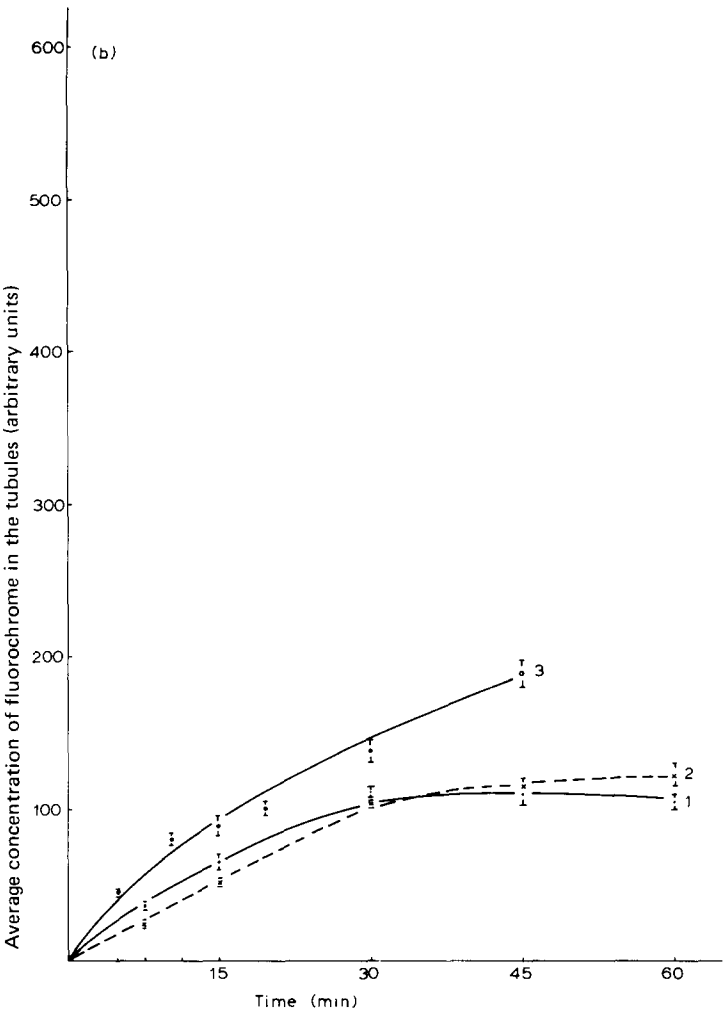


Fig. 1. Time course of fluorescein and uranin uptake by proximal tubules in normal (113.4 mM of Na^+) and in Na^+-free media with substitution of NaCl by sucrose (a), choline chloride and LiCl (b). (a) Fluorescein (1) and uranin (2) in medium with normal Na^+ concentration; fluorescein (3) and uranin (4) in medium with NaCl substituted by sucrose. (b) Fluorescein (1) and uranin (2) in medium with NaCl substituted by choline chloride; fluorescein (3) in medium with NaCl substituted by LiCl. Concentration of fluorescein and uranin in the medium is $5 \cdot 10^{-5}$ M. The vertical lines show the 95% confidence limits for each value.

Uranin transport in Na^+-free medium was inhibited by *p*-aminohippurate (the concentration ratio of *p*-aminohippurate/uranin is 80) by 46%. Strophantin K ($5 \cdot 10^{-5}$ M) inhibits fluorescein uptake after 30 min by 20% in the choline chloride medium and by 54% in the normal medium. Visual control shows that the action of both these agents causes the lowering of fluorescein concentration in the cytoplasm and in the tubules lumen.

A special series of experiments was performed to study the influence of preincubation of a surviving kidney for 60 min in Na^+-free (choline chloride) medium on the fluorescein transport. The preincubation was made to remove

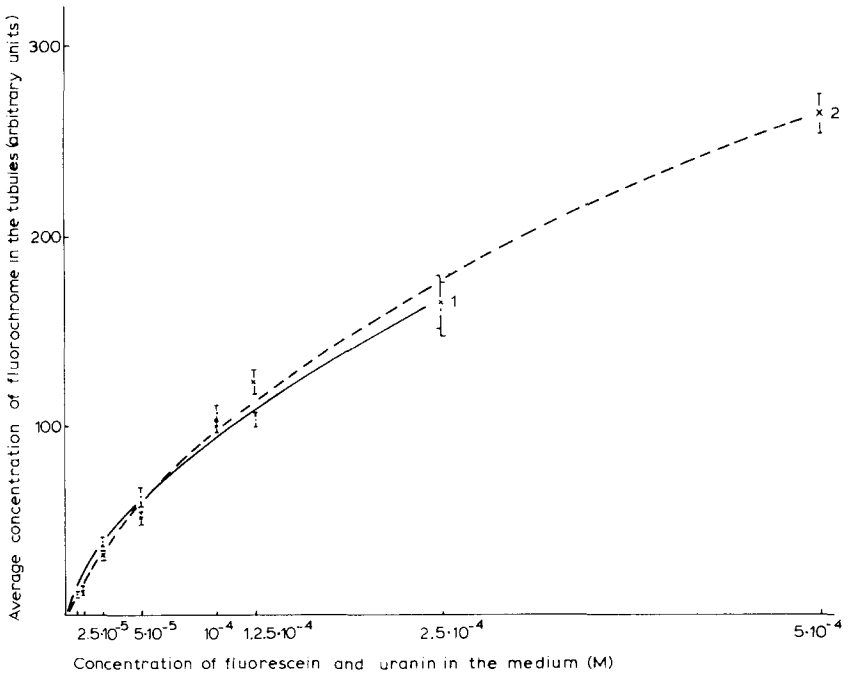


Fig. 2. Uptake of fluorescein (1) and uranin (2) in proximal tubules with their different concentrations in the Na^+-free (choline chloride) bath medium. Time of incubation, 15 min.

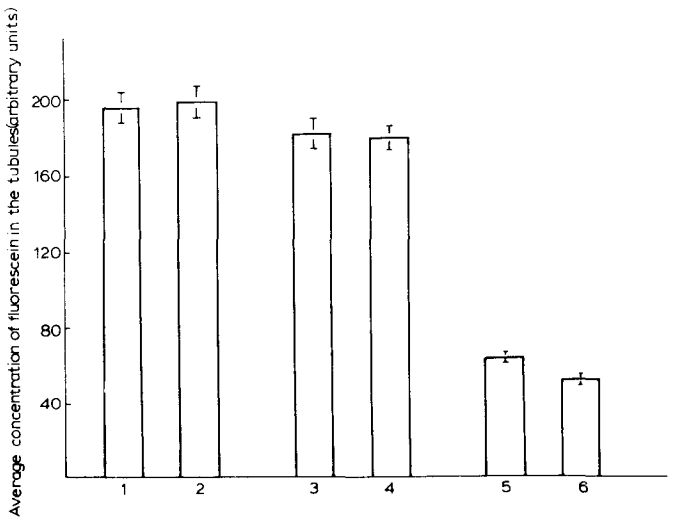


Fig. 3. Influence of preincubation in normal (113.4 mM of Na^+) and Na^+-free media on the fluorescein uptake in proximal tubules. (1) Incubation with fluorescein in normal medium without preincubation. (2) As in (1), but with preincubation for 60 min in normal medium. (3) Incubation with fluorescein in normal medium with preliminary perfusion of tubular lumens by normal medium, without preincubation. (4) As in (3), but with preincubation for 30 min in Na^+-free medium. (5) Incubation with fluorescein in Na^+-free medium with preliminary perfusion of tubular lumens by Na^+-free medium, without preincubation. (6) As in (5), but with preincubation for 60 min in Na^+-free medium. Time of incubation 15 min. Concentration of fluorescein in incubation medium is $5 \cdot 10^{-5}$ M.

TABLE I

APPARENT MICHAELIS CONSTANTS (K_m), MAXIMAL RATES (V) AND V/K_m RATIOS FOR FLUORESCEIN AND URANIN TRANSPORT IN THE PRESENCE AND IN THE ABSENCE OF Na^+ IN THE BATH MEDIUM

Conditions of incubation	K_m ($\times 10^{-4}$ M)	Confidence limits for K_m ($\times 10^{-4}$ M)	V *	Confidence limits for V *	V/K_m *	Confidence limits for V/K_m *
Uranin in the medium with normal Na^+	0.90	0.64—1.54	41.5	±10.4	46.1	± 8.2
Uranin in Na^+-free medium (choline-chloride)	2.74	2.06—4.06	26.0	± 4.5	9.5	± 1.5
Net Na^+-dependent uranin transport	0.68	0.47—1.23	25.4	± 6.3	37.4	± 8.0
Fluorescein in the medium with normal Na^+	1.08	0.64—3.52	44.4	±18.5	41.1	±12.0
Fluorescein in Na^+-free medium (choline-chloride)	1.34	1.04—1.88	16.2	± 2.4	12.1	± 1.8
Net Na^+-dependent fluorescein transport	0.95	0.51—6.45	29.1	±14.0	30.6	±11.8
Fluorescein in Na^+-free medium (LiCl)	1.20	0.80—2.20	26.2	± 66.	21.8	± 5.2

* The values are presented in working units.

Na^+ from peritubular zone, where these ions come from the cells of proximal tubules. The results of these experiments (Fig. 3) show that such preincubation inhibits significantly (by 12%) fluorescein transport in the Na^+-free medium only (compare columns 5 and 6).

The apparent transport constants K_m and V were computed for fluorescein transport in the Na^+-free medium after preincubation in this medium for 60 min (test) and without preincubation (control). They are $K_m = 2.5 \cdot 10^{-4}$ M (from $1.6 \cdot 10^{-4}$ M to $5.0 \cdot 10^{-4}$ M) $V = 23.4 \pm 8.0$ of working units/min (test) and $K_m = 2.0 \cdot 10^{-4}$ M (from $1.4 \cdot 10^{-4}$ M to $3.7 \cdot 10^{-4}$ M), $V = 24.4 \pm 7.3$ of working units/min (control). Thus, the preincubation causes an increase of K_m by 25% with unchanged V, although the change of K_m is not significant.

It was shown in our experiments that fluorescein run out from proximal tubules preloaded with this acid is accelerated by choline chloride medium in comparison with the normal medium (Fig. 4a). Addition of *p*-aminohippurate to the medium accelerated sharply the run out of fluorescein in the normal medium, the maximum of effect was reached after 15 min of incubation (Fig. 4b). In Na^+-free medium *p*-aminohippurate had a lesser effect on the fluorescein run out as compared to the normal medium and the maximum of effect was reached after 30 min of incubation (Fig. 4c).

Dependencies of the fluorescein and uranin transport rate on the concentration of a corresponding substance in the medium were investigated in media

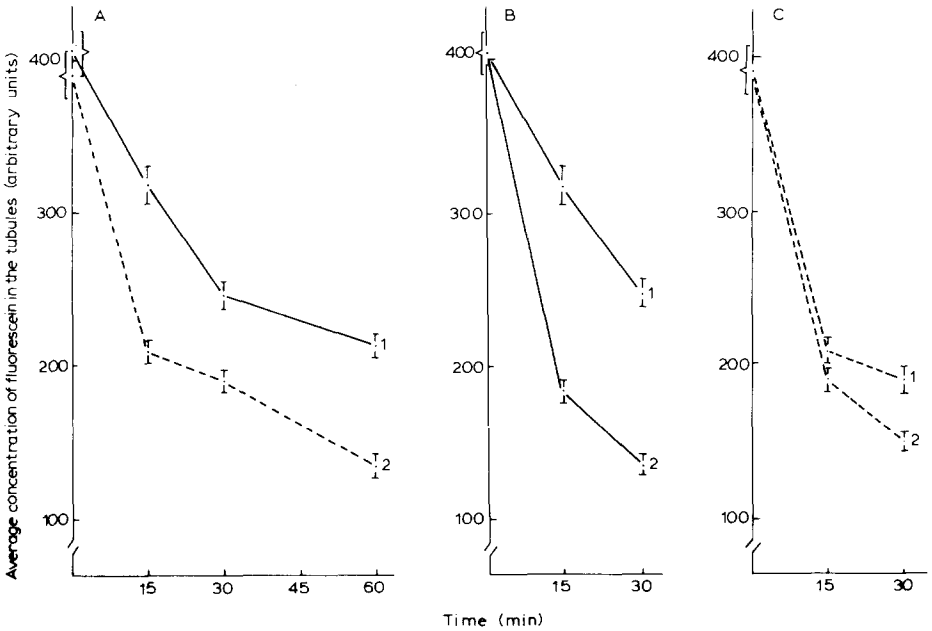


Fig. 4. Action of Na^+-free medium and *p*-aminohippurate on the fluorescein run out from the proximal tubules preloaded with this dye. (A) Fluorescein run out into normal (1) and Na^+-free (2) medium. (B) Fluorescein run out into normal medium in the absence (1) and in the presence (2) of *p*-aminohippurate. (C) Fluorescein run out into Na^+-free medium in absence (1) and in the presence (2) of *p*-aminohippurate. Concentration of *p*-aminohippuarte in fluorescein-free media is 10^{-3} M.

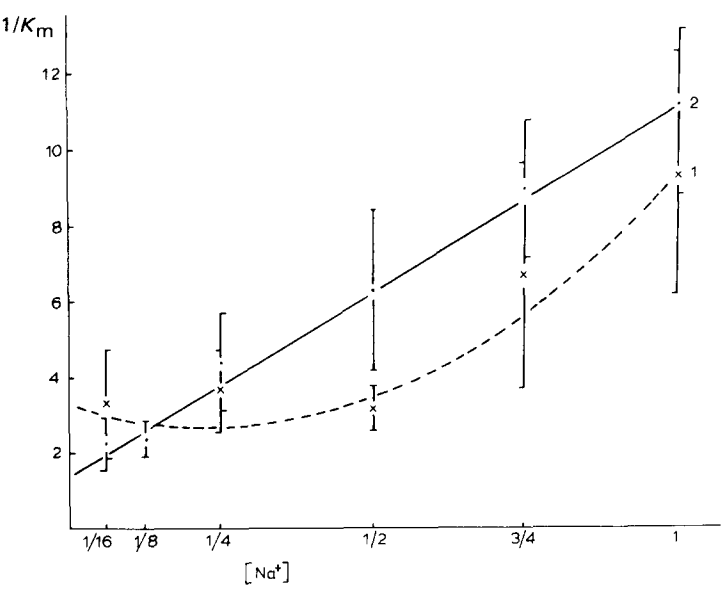
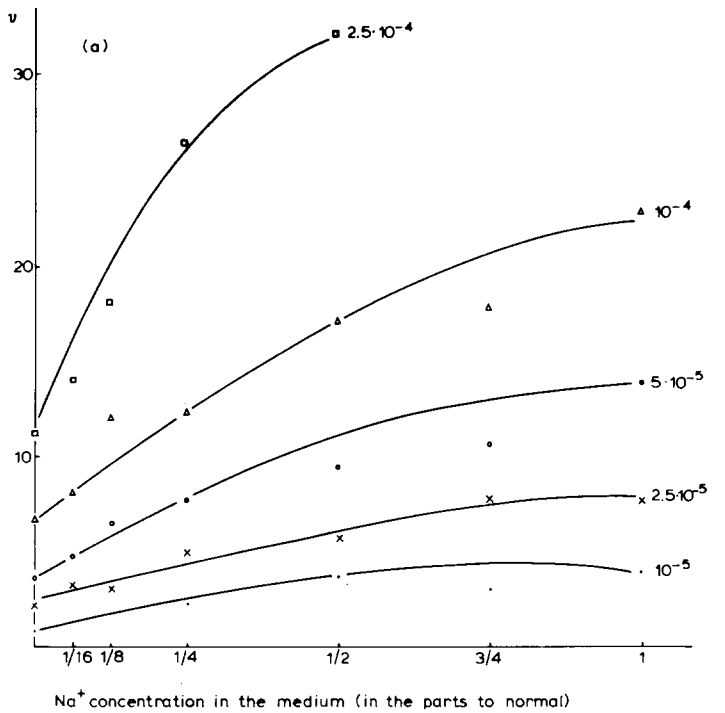


Fig. 5. Dependence of $1/K_m$ value for fluorescein (1) and uranin (2) transport into proximal tubules on the Na^+ concentration in the bath medium. The vertical lines show the 95% confidence limits for each value.



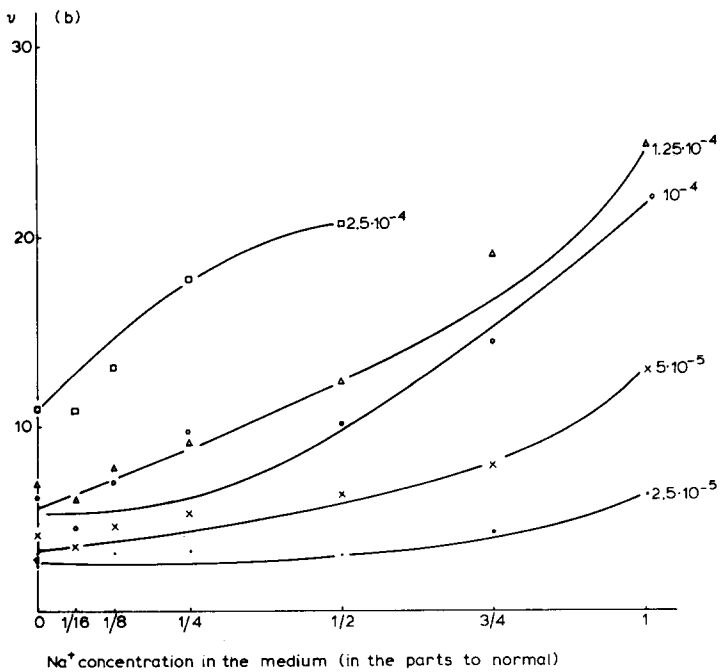


Fig. 6. Dependence of uranin (a) and fluorescein (b) transport rate (v) on the Na^+ concentration on the bath medium. The figures near the curves show fluorescein and uranin concentration (M) in the medium.

that contained different but constant Na^+ concentrations. The Na^+ concentrations used were as follows: normal (1) or 3/4, 1/2, 1/4, 1/8 and 1/16 of the normal concentration. All experimental curves describing these dependencies had the form of a hyperbolia. Visual control showed that fluorescein and uranin uptake in the lumen of tubules occurred, but it was much weakened in media with low Na^+ concentrations. The data recalculated by the Lineweaver-Burk method correlate well with the straight line (correlation coefficient was not below 0.94). The apparent K_m and V and the V/K_m ratio for uranin and fluorescein transport determined by solution of Lineweaver-Burk equation are presented in Table II. It is seen that in the media with low Na^+ concentration K_m for fluorescein transport exhibits a tendency to increasing, although the differences in K_m are not significant. But the V/K_m ratio decreases significantly under these conditions. In the case of uranin augmentation of K_m and significant decrease of the V/K_m ratio are not followed by a change of V in a similar way.

Dependence of $1/K_m$ on the Na^+ concentration in the media used (Fig. 5) is linear for uranin transport (correlation coefficient is 0.993) and essentially nonlinear for fluorescein transport (correlation coefficient is 0.436). The latter

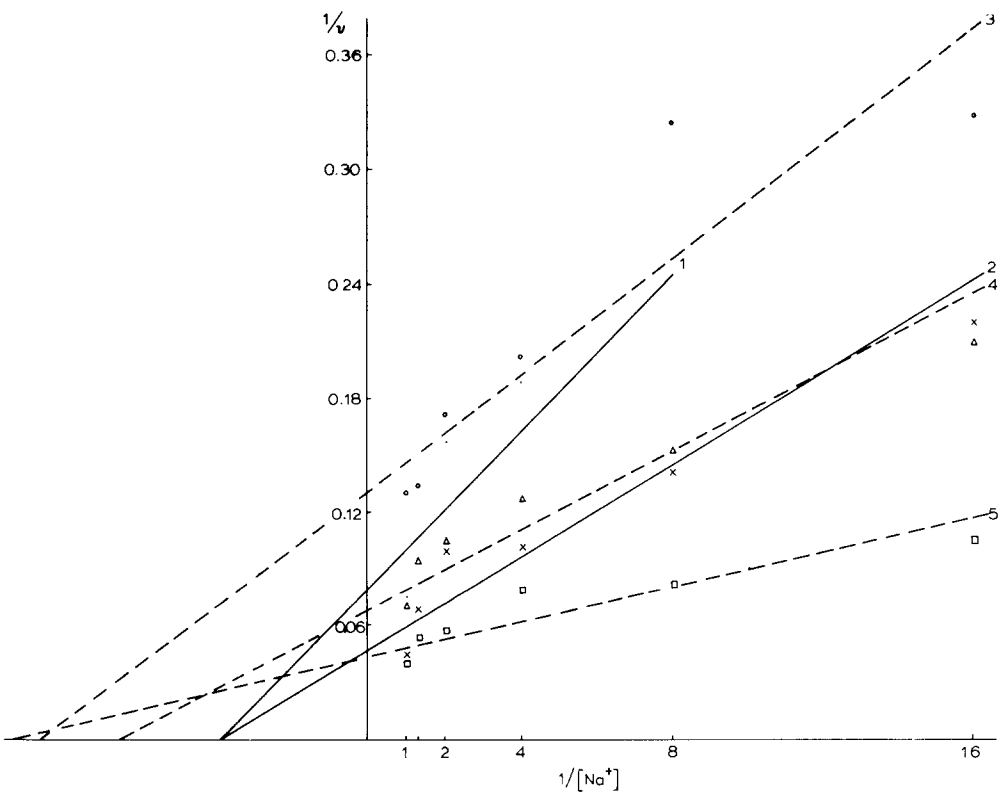


Fig. 7. Lineweaver-Burk plots for dependence of reversal rate of fluorescein and uranin transport into proximal tubules on the reversal Na^+ concentration in the bath medium. 1 and 2, fluorescein concentration in the medium is $5 \cdot 10^{-5}$ M and 10^{-4} M, respectively. 3, 4 and 5, uranin concentrations in the medium is $2.5 \cdot 10^{-5}$ M, $5 \cdot 10^{-5}$ M and 10^{-4} M, respectively.

TABLE II

DEPENDENCE OF MICHAELIS-MENTEN EQUATION PARAMETERS FOR FLUORESCEIN AND URANIN TRANSPORT INTO PROXIMAL TUBULES ON THE N_a^+ CONCENTRATION IN THE BATH MEDIUM

Fluorescein							Uranin					
Na^+ concentration in the parts to normal one (113.4 mM)	K_m ($\times 10^{-4}$ M)	Confidence limits for K_m ($\times 10^{-4}$ M)	V *	Confidence limits for V *	V/K_m *	Confidence limits for V/K_m *	K_m ($\times 10^{-4}$ M)	Confidence limits for K_m ($\times 10^{-4}$ M)	V *	Confidence limits for V *	V/K_m *	Confidence limits for V/K_m *
1/16	2.91	1.54—25.0	21.9	±13.1	7.5	± 2.1	4.32	2.53—14.9	49.5	±21.8	11.5	±5.5
1/8	—	—	—	—	—	—	4.11	2.90—7.09	56.8	±14.2	13.8	±2.5
1/4	2.77	1.71—7.19	35.8	±14.2	12.9	± 2.9	2.28	1.49—4.78	47.4	±15.6	20.8	±4.2
1/2	3.09	2.32—4.59	43.9	±10.0	14.2	± 3.3	1.59	0.94—5.24	48.5	±19.5	30.5	±9.7
3/4	1.52	0.78—3.33	38.9	±11.5	25.6	± 9.2	1.11	0.78—1.92	37.2	±10.2	33.5	±5.0
1	1.08	0.64—3.52	44.8	±18.5	41.5	±12.0	0.90	0.64—1.54	41.5	±10.4	46.1	±8.2

* The values are presented in working units.

TABLE III

MICHAELIS-MENTEN EQUATION PARAMETERS FOR ORGANIC ACIDS TRANSPORT INTO PROXIMAL TUBULES WITH VARYING Na^+ CONCENTRATION AND CONSTANT SUBSTRATE CONCENTRATION IN THE MEDIUM

Substrate	Substrate concentration (M)	K_{mNa^+} (mM/l)	Confidence limits for K_{mNa^+} (mM/l)	V_{Na^+} *	Confidence limits for V_{Na^+} *	Coefficient of correlation between $1/\overline{V}$ and $1/[Na^+]$	Coefficient of correlation between $1/\overline{V}$ and $1/[Na^+]^2$
Fluorescein	$5 \cdot 10^{-5}$	28.7	14.7—189.4	12.3	4.5	0.923	0.814
Fluorescein	10^{-4}	28.9	17.0— 81.6	20.9	5.3	0.996	0.771
Uranin	$2.5 \cdot 10^{-5}$	13.2	8.4— 31.0	7.9	1.2	0.916	0.801
Uranin	$5 \cdot 10^{-5}$	16.4	10.4—379.9	14.1	2.0	0.918	0.775
Uranin	10^{-4}	12.2	7.4—351.5	22.2	2.8	0.883	0.739

* The values are presented in working units.

curve looks like a parabola and the dependence of $1/K_m$ for fluorescein transport on the square concentration of Na^+ has the correlation coefficient 0.708.

Then we analyzed the dependence of fluorescein and uranin transport rates on the concentration of Na^+ in the media with a constant concentration of these substrates. Appropriate curves were plotted for media containing 5 different constant concentrations of fluorescein or uranin. From Fig. 6a it is seen that all curves for uranin transport look like a hyperbola while those for fluorescein transport are less typical. Besides it was established that the action of Na^+ in the medium on the fluorescein and uranin transport is greater in the media with a high constant concentration of these substances. The dependencies described above may be represented in the form of Lineweaver-Burk plots (Fig. 7); all experimental points are lying on the straight lines. Solution of the appropriate Lineweaver-Burk equation gave the apparent K_{mNa^+} and V_{Na^+} for fluorescein and uranin transport (Table 3). The apparent K_{mNa^+} value both for fluorescein and for uranin does not depend on the substrate concentration in the medium, while V_{Na^+} decreases proportionally to the decrease of a substrate concentration in the medium.

It is curious that the mean apparent K_{mNa^+} for fluorescein transport (28.8 mM) is exactly two times greater than that for uranin (13.9 mM). The K_{mNa^+} value is evidently equal to the Na^+ concentration in the medium which gives a half-maximal rate of the substrate transport under such conditions. Thus, for reaching a half-maximal rate of fluorescein transport in the tubules the Na^+ concentration in the medium should be two times greater than for the case of uranin transport.

Discussion

The method used for measuring the total luminescence of a marker organic acid simultaneously in the epithelium and in the lumen of proximal tubule segment permits determination of the whole amount (in working units) of a substance transferred across basal membrane during incubation. Therefore, the transport constants (K_m and V) obtained in our experiments refer directly to the basal membrane [22]. That is why we discuss the transport of organic acids in proximal tubule rather than in lumen of the tubule. In the process of active transport an organic acid is transferred across the basal membrane from the medium into the epithelium of proximal tubule, and then it goes across the apical membrane from the epithelium into the lumen. After 5 min of incubation the intracellular concentration of fluorescein remains at a constant level while its concentration in the lumen depends linearly on the time of incubation. Consequently, the intensity of luminescence of a whole tubule may be attributed mainly to the marker fluorochrome in the lumen of the tubule. However, if organic acid transport across apical membrane of the tubule is somehow inhibited the contribution of intracellular fluorochrome to the total intensity of luminescence may increase markedly [22]. The question arises whether fluorescein (uranin) binds to cytoplasmic proteins, and if it is the case how the binding may influence the accuracy of microfluorimetric measurements.

Our visual observations in experiments with short-term incubation (1—2

min) show that fluorescein (uranin) spreads as rapidly along the tubular cell as it runs completely out. This fact suggests the absence of any appreciable binding of fluorescein (uranin) to cellular proteins. We expect to obtain a stronger evidence in favour of this assumption by determining the spectra and polarization of luminescence of these substances in the cell interior. Preliminary data allow the consideration of the intracellular binding without influencing the accuracy of fluorimetric determination.

The obtained results show that in the effect of Na^+-deficiency in the medium there are certain regularities common for both transport substrates (fluorescein and uranin) as well as differences (mainly quantitative) between these substances. The main common effect of the absence or decrease of Na^+ in the bath medium is the inhibition of transport of the both marker anions into proximal tubules. In the normal medium the transport rate remains constant for 45 min whereas with the substitution of NaCl by sucrose or choline chloride it lowers immediately and constantly reaching the zero after 45—60 min of incubation. This effect of complete stopping of the transport is most strongly pronounced for fluorescein in sucrose medium and both for fluorescein and for uranin in choline chloride medium. With the substitution of NaCl by LiCl the rate of fluorescein transport into tubules lowers more slowly as compared with the substitution of NaCl by sucrose or choline chloride. A similar action of substitution of NaCl by LiCl was observed by Hoshi and Hayashi [10] when these authors studied the phenol red transport by a surviving kidney of goldfish.

The kinetic analysis shows (Table II) that inhibition of fluorescein and uranin transport rate in proximal tubules when Na^+ in the medium is lowering is caused by progressed augmentation of the apparent transport K_m. Although in the case of fluorescein a difference even between the extreme K_m values is not significant, the K_m augmentation is obvious because the V/K_m ratio decreases significantly with unchanged V. When Na^+ is completely absent from the medium (choline chloride medium), along with the augmentation of K_m, V decreases (Table I). For understanding the basis of these changes it should be remembered that the apparent transport $K_m = (K_{-1} + K_2)/K_{+1}$, where K_{+1} is the rate constant of formation a carrier-substrate complex (PS), K_{-1}, the rate constant of this complex dissociation, K_2 its translocation rate. K_m is not identical to the dissociation constant (K_s), but is related to it as follows: $K_m = K_s + K_2/K_{+1}$. Thus the K_m value depends on the affinity between a carrier and a substrate and on the translocation rate of complex PS. The maximal transport rate $V = K_2[P]$, where [P] is carrier concentration in the membrane. We assume that in our short-term experiments [P] is a constant value. Consequently, V changes may be induced only by K_2 change, while an increase of K_m with unchanged V may be the results of K_{-1} and K_1 changes (i.e. at the expense of alteration in the affinity between a carrier and a substrate). After complete substitution of NaCl by choline chloride K_{-1}, K_{+1} and K_2 (i.e. both the affinity and the translocation rate) change. When Na^+ is substituted by Li^+ the V value is higher in comparison with the case of substitution of Na^+ by choline$^+$ with unchanged K_m. Thus, it is obvious that Li^+ does not influence the affinity between a carrier and a substrate and substitution of Na^+ by Li lowers the translocation rate of a transport complex for a lesser extent than substitution of Na^+ by choline$^+$. In the Na^+-dependent medium the K_m increase regarded as a change

(lowering) of an affinity between a carrier and a substrate is interpreted by most investigators as a proof of direct drawing of Na^+ into the formation of a transport complex [1—10]. Thus we may suppose that Na^+ from the medium takes part in formation of a transport complex for fluorescein and uranin anions transfer and it cannot be substituted by choline$^+$ or Li^+. Transport of organic acids in proximal tubules of the grass frog kidney is typically Na^+-dependent. This fact is in good agreement with Vogel and Kröger's evidence for the lake frog [16] and with Hoshi and Hayashi's observation on the goldfish [10].

Visual control of fluorescein and uranin transport in the Na^+-free (sucrose or choline chloride) media shows that maximal concentration of these substances which is attained after prolonged incubation in the tubules is equal to or slightly exceeds their concentration in the medium. Thus a transport in Na^+-free medium stops being active. However, it is important to note that in spite of the removal of Na^+ from the tubular lumen and from capillaries by perfusion some amount of Na^+ is likely to be present in the peritubular region due to extrusion of this ion from tubular cells by (Na^+, K^+)-ATPase. A slightly inhibitory effect of strophantin (specific inhibitor of (Na^+, K^+)-ATPase) on the fluorescein transport in Na^+-free medium and an inhibitory effect of prolonged preincubation in Na^+-free medium (i.e. washing out of Na^+) allowed for this assumption. It is of interest that the inhibitory effect of washing clean on the fluorescein transport is related to the increase of K_m, i.e. to the decrease of affinity between a carrier and a substrate. Thus, in Na^+-free medium without prolonged preincubation a portion of fluorescein and uranin (about 20%) is accumulated at the expense of Na^+ in the peritubular region.

So the organic acids transport into proximal tubules may be divided into two parts: Na^+-dependent active transport and Na^+-independent nonactive transport. Na^+-independent transport (in the Na^+-free medium), however, exhibits the same characteristic features of the carrier involvement as the transport in normal medium, a kinetics with saturation, competitive inhibition of the uptake and acceleration of the run out of fluorescein from tubules preloaded with this acid by another organic acid in the medium. Analogous data are usually treated as an indication of a share of specific mobile carrier in the transport process [31—34]. Consequently, with the presence of Na^+ in the medium the carrier fulfils an active transport, whereas in the absence of Na^+ it performs only facilitated diffusion of organic acids. Contribution of Na^+-dependent and Na^+-independent components into total process of substance transfer depends on the Na^+ concentration in the medium. Therefore, the value determined with the given Na^+ concentration in the medium may be corrected. Table IV presents the dependence of the apparent K_m for the net Na^+-dependent uranin transport on the Na^+ concentration in the medium. As the Na^+ concentration in the medium decreases 16 times K_m increases 40 times as compared to a 4-fold increase of K_m in the case of uncorrected data.

For a carrier to fulfill an active transport the inequality of its affinities to a substrate on the opposite sides of the membrane must be provided. Since the affinity of an organic acid carrier depends on the Na^+ concentration, this claim is satisfied in the normal medium. In fact, on the outer side of the membrane (Na^+-rich) a carrier has a high affinity to a substrate. After translocation of the complex to the inner side of the membrane (Na^+-poor) an affinity of a carrier

TABLE IV

MICHAELIS-MENTEN EQUATION PARAMETERS FOR NET Na^+-DEPENDENT URANIN TRANSPORT INTO PROXIMAL TUBULES

Net Na^+-dependent transport rates were determined as a difference between the rate of uranin transport in the medium with given Na^+ concentration and that in Na^+-free medium with the same uranin concentration.

Na^+ concentration in the medium in the parts to normal one (113.4 mM)	K_m $\times 10^{-4}$ M	Confidence limits for $K_m \times 10^{-4}$ M	V *	Confidence limits for V *	V/K_m *	Confidence limits for V/K_m *
1/16	26.4	6.67—120.5	72.5	±18.6	2.7	±1.1
1/8	4.74	2.55—33.3	27.6	±14.3	5.8	±2.0
1/4	2.75	1.51—14.7	30.4	±16.0	11.1	±3.4
1/2	1.28	0.68—11.5	28.6	±13.3	22.3	±10.3
3/4	0.86	0.57—1.79	20.4	±6.3	23.7	±4.7
1	0.68	0.47—1.23	25.4	±6.3	37.4	±8.0

* The values are presented in working units.

to substrate decreases and the complex dissociates. The additional proof of the reality of such a mechanism is a drastic acceleration of fluorescein run out from tubules into Na^+-free medium.

A number of investigators consider that on the basis of a dependence of K_m on the Na^+ concentration in the medium it is possible to determine the number of Na^+ participating in the formation of a transport complex [2,10]. Such an approach based on the peculiarities of the enzyme reaction with two substrates or with a substrate and an activator is open for discussion. It was shown [35] that if an enzyme reaction with two substrates involves the formation and dissociation of an enzyme-substrate complex by two compulsory pathways one may assume a ratio of rate constants of separate reaction stages when the summary rate would be at the same time proportional to the concentration of the first substrate and to the square of the second substrate concentration, that is why Wheeler and Christensen [36] consider that the occurrence of a quadratic dependence of the $1/K_m$ value on the Na^+ concentration does not prove the formation of a transport complex with two Na^+.

As mentioned above Hoshi and Hayashi [10] discovered for phenol red transport a quadratic dependence of the $1/K_m$ value on the Na^+ concentration in the medium. They interpreted it as evidence supporting the formation of a 4-fold transport complex: a carrier plus a substrate plus 2 Na^+. We observed a similar dependence for fluorescein transport (the structure of fluorescein and phenol red are very much alike). For uranin transport the $1/K_m$ value was found to be dependent linearly on the Na^+ concentration. The difference between the fluorescein and uranin anions transport is confirmed by the fact that the K_{mNa^+} value for fluorescein transport is exactly twice as large as that for uranin transport. In other words for a transfer across the membrane a fluorescein anion requires two Na^+, whereas for uranin anion transport only one Na^+ from the medium is necessary. The existence of similar differences between the fluorescein and uranin transport may not be explained on the basis of Wheeler and Christensen's kinetic model [36]. In what way do the ionic forms of fluorescein and uranin differ from each other in salt media? In terms of the theory of

protolytic equilibrium [24—26] fluorescein and uranin in solutions exist in forms of mon- and dianions. However, fluorescein is not known to be dissolved in distilled water, but it is dissolved in weak alkaline solutions, i.e. in solutions which contain Na^+ or NH_4 [25]. Uranin (disodium salt of fluorescein) exhibits good solubility even in distilled water. Evidently, it is advisable to consider the fluorescein and uranin state in solution in the framework of Lewis' theory as a formation of coordination compounds between Lewis' acid (Na^+, NH_4, choline$^+$) and Lewis' base (fluorescein, uranin) [37,38]. In this case, after dissolution of fluorescein and uranin in a medium with high Na^+ concentration there is no difference between the forms of fluorescein and uranin molecules. They are similar and represent a coordination compound of fluorescein with Na^+. In fact, fluorescein transport in proximal tubules did not differ from uranin transport when the media with a normal Na^+ concentration were used. If the medium does not contain Na^+ (it is substituted by choline$^+$ or Li^+) fluorescein in solution may form coordination compounds with choline$^+$ (or Li^+) only, while uranin may coordinate with both choline$^+$ (or Li^+) and Na^+. The ratio between the concentrations of these coordination compounds will be defined by their formation constants. In utmost simplification it may be said that distinction between the fluorescein and uranin monoanions is that in Na^+-free medium the latter contains Na^+ instead of H of a hydroxyl group. Therefore, it may be assumed that in an active transport complex of a carrier with fluorescein and uranin monoanions two Na^+ are contained, but in the case of uranin only one Na^+ comes from the medium. The number of Na^+ (one or two) participating in organic acids transport is determined evidently by the organic anion structure. It is not unlikely that one Na^+ may be substituted by an aminogroup. If this is the case, only one Na^+ from the medium is necessary for the *p*-aminohippuric anion active transport into renal proximal tubules of the frog.

In conclusion it must be remined that Gerencser and Hong [19] who worked with the rabbit kidney cortex slices did not detect any influence of Na^+ in the medium either on the run out of *p*-aminohippurate from slices or on the affinity between a carrier and a substrate. These data are at such variance with our results and with the evidence of other investigators who worked on intact kidney of cold-blooded animals [10,16] that their verification on the more intact preparation of mammal's kidney is urgently called for. Such verification would enable us to solve the question of similarity or of difference between active transport mechanisms for organic acids in cold-blooded animals and mammals.

References

1 Crane, R.K. (1965) Federat. Proc. 24, 1000—1006
2 Vidaver, G. (1964) Biochemistry 3, 662—667
3 Schultz, S. and Curran, P. (1970) Physiol. Rev. 50, 637—718
4 Schultz, S. and Curran, P. (1974) in Current Topics in Membranes and Transport, Vol. 5, pp. 225—281
5 Christensen, H.N. (1972) in Role of Membranes in Secretory Processes (Bolis, L., Keynes, R. and Willbrandt, W., eds.), pp. 433—447, North-Holland Publishing Co., Amsterdam
6 Heinz, E. (1972) in Na^+-linked Transport of Organic Solutes (Heinz, E., ed.), pp. 15—19.Springer Berlin
7 Heinz, E. (1974) in Current Topics in Membranes and Transport, Vol. 5, pp. 137—159
8 Eddy, A. (1972) in Na^+-linked Transport of Organic Solutes, pp. 28—38
9 Schafer, J. (1972) Ibid. pp. 68—82

10 Hoshi, T. and Hayashi, H. (1970) Japan J. Phyisol. 20, 683—693
11 Csaky, T. (1963) Federat. Proc. 22, 3—8
12 Kimmich, G. (1970) Biochemistry 9, 3669—3678
13 Kimmich, G. (1972) in Na^+-linked Transport of Organic Solutes, pp. 116—129
14 Kimmich, G. and Randless, J. (1975) J. Membrane Biol. 33, 57—76
15 Pitts, R. (1974) Physiology of the Kidney and Body Fluids, Year Books Bed. Publishers, Chicago
16 Vogel, G. and Kroger, W. (1965) Pflugers Arch. 286, 317—322
17 Bresler, V., Bresler, S. and Nikiforov, A. (1975) Biochim. Biophys. Acta 406, 526—537
18 Bresler, V.M. and Natochin, Y.V. (1973) Bull. Exper. Biol. Med. 75, 67—69 (In Russian)
19 Gerencser, G. and Hong, S. (1975) Biochim. Biophys. Acta 406, 108—119
20 Bresler, V.M. and Nikiforov, A.A. (1974) Dokl. Akad. Nauk SSSR, 219, 227—230 (in Russian)
21 Bresler, V.M. and Kachman, A.N. (1975) Lab. Delo 2, 109—111 (in Russian)
22 Bresler, V.M., Kachman, A.N. and Nikiforov, A.A. (1976) Z. Evol. Bioch. Physiol. 12, 120—127 (in Russian)
23 Murthy, L. and Foulkes, E. (1967) Nature 213, 180—181
24 Zanker, V. and Peter, W. (1958) Chem. Berichte 91, 572—580
25 Rozwadowski, M. (1961) Acta Phys. Polonica 203, 1005—1017
26 Adamov, V.S. and Kandargan, L.T. (1961) Optika i Spectroskop 11, 419—422 (in Russian)
27 Bresler, V. and Nikiforov, A. (1977) Tsitologia 19, 82—89 (in Russian)
28 Sernetz, M. and Thaer, Q. (1975) in Fluorescence Techniques in Cell Biology (Thaer, Q. and Sernety, M., eds.), pp. 41—50, Springer, Berlin
29 Rumshisky, L.S. (1970) Mathematical Treatment of Experimental Results Moskow (in Russian)
30 Jervis, E. and Smith, D. (1959) J. Physiol. 149, 433—441
31 Willbrandt, W. and Rosenberg, T. (1961) Pharmacol. Rev. 13, 109—184
32 Rosenberg, T. and Willbrandt, W. (1963) J. Theoret. Biol. 5, 288—305
33 Stein, W. (1967) The Movement of Molecules Across Cell Membranes, Academic Press, New York
34 Kotyk, A. and Janacek, K. (1970) Cell Membrane Transport. Principles and Techniques, Plenum Press, New York
35 Ferdinand, W. (1966) Biochem. J. 98, 278—283
36 Wheeler, K. and Christensen, H. (1967) J. Biol. Chem. 242, 3782—3788
37 Bailar, J. (1956) Chemistry of the Coordination Compounds, Reinhold, New York
38 Basolo, F. and Johnson, R. (1964) Coordination Chemistry, Benjamin, New York