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STRUCTURE AND ACTIVE TRANSPORT IN THE PLASMA MEMBRANE OF THE TUBULES OF FROG KIDNEY

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SUMMARY

The active transport of organic anions through the plasma membrane of the proximal tubules of frog kidney was studied. For this purpose a marker anion, fluorescein, was used, its flow into the tubules registered by the increase of fluorescense. The kinetics of transport was measured as function of time, concentration of substrate, concentration of a competing acid (*p*-aminohippuric acid) and temperature. The process is inhibited by strophantin, a specific poison for $(Na^+ + K^+)$-dependent ATPase. These data show that fluorescein transport is effected with the participation of a charged carrier, probably by the downfield mechanism postulated by Mitchell. To confirm this mechanism, a passive flow of K^+ was created inwards across the membrane of the proximal tubules by means of valinomycin. It led to the discharge of the membrane and to the inhibition of fluorescein transport. Anions are transported downfield across the membrane, probably in a state of complexes with two Na^+ ions.

A magnetic field of 10 000–28 000 oersted inhibits the fluorescein transport strongly. This can be regarded as a proof of the liquid-crystalline structure of biological membranes and demonstrates the importance of this structure for active transport.

INTRODUCTION

The plasma membranes in the internal organs of animals contain special enzymatic systems related to the active transport of water-soluble ions and metabolites across the hydrophobic barrier, the lipid double layer [1–5]. The molecular mechanism of these processes is mostly unknown. But all these systems of transport reveal many characteristic features. (1) They effect the transport of substances against a concentration gradient. (2) They show specificity for the chemical structure of transported substances and competitive inhibition by structural analogues. (3) They depend on energy sources. (4) The process is temperature dependent, like enzymatic catalysis.

The theoretical basis of modern views on the subject is given by Mitchell's theory [6–8], which explains the active transport of different substances by the

movement of their ionized complexes in an electrochemical gradient. The latter is generated in the plasma membrane by an ATPase-dependent sodium pump [6-8]. Hence the driving force of active transport is the electric field. As inner side of the plasma membrane is negatively charged, it is puzzling that organic acids move downfield as if they were positively charged. We shall come back to this question later.

An interesting object for the study of membrane transport is the kidney. One of its main functions is the transport of organic acids from blood and tissue fluids into the cellular cytoplasm across the basal membrane and then further into the lumen of renal proximal tubules across the apical membrane. The concentration of acids inside the tubules is much higher than in the surrounding cells [3]. It was shown earlier [9] that this process can be visualized if a surviving kidney is immersed into a solution of fluorescein. In this case the dye serves as marker acid. It is readily transported and concentrated inside the kidney tubules, and its concentration can be measured by means of a microfluorimeter. A special microscope was designed for this purpose [10, 11] supplied with a contact objective lens, which enables the optics to be focused on a single tubule without damaging the kidney. The image of a single tubule is observed visually and is simultaneously projected on the cathode of a photomultiplyer tube. The fluorescense of the dye inside a tubule increases linearly with time and this gives a measure of transport kinetics [11]. The results of such measurements are presented in this paper.

MATERIALS AND METHODS

For in vitro experiments we used the surviving kidneys of male frogs *Rana temporaria*. The kidneys were excised before each experiment and washed in Ringer's solution. The latter contained 111 mM NaCl, 3.35 mM KCl, 2.8 mM $CaCl_2$, 2.38 mM $NaHCO_3$, 5.5 mM glucose. It was established that frogs kidneys do not require special oxygenation during the 30 min time of our experiments. No difference in the kinetics of active transport was found if the Ringer solution was continuously aerated.

All solutions of fluorescein, *p*-aminohippuric acid, valinomycin and strophantin were prepared with the same Ringer solution. The temperature of the solution was usually 18–19 °C, pH 7.4. One kidney was incubated in the fluorescein solution, the second (the control) in the Ringer solution to measure the background fluorescence of proteins and other substances in the tubules. After incubation in a given solution, the kidney was rinsed with Ringer's solution and transferred to the cell of the microfluorimeter. The measuring device is shown in principle on Fig. 1. It consists of a microscope with a contact objective lens 25×0.75. Illumination of the object was effected through the same objective lens. A stabilized iodine lamp was used as light source and an excitation filter with a transmission maximum at 400 nm produced the light beam for the excitation of fluorescence. In the lightpath of the observation system a barrier filter with a maximum of transmission at 540 nm was inserted. The diameter of the measuring diaphragm in the plane of the object was 60 μm. It corresponded to the average width of the proximal tubules. The background fluorescence of the tubules was always subtracted from the data (it was a small correction not exceeding a few percent). The fluorescent light that was emitted within the area of the photometric probe, coming from the lumen of the tubule as well as the cytoplasm of

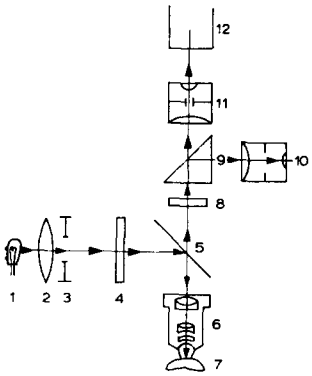

Fig. 1. Scheme of the microfluorimeter for measurements on a whole surviving kidney. 1. iodine lamp with stabilized direct current feed; 2, condenser lens; 3, diaphragm; 4, excitation filter, 5, beam-splitting plate; 6, contact objective; 7, object; 8, barrier filter; 9, prism for visual observation; 10, ocular lens; 11, ocular of the objective photometer with measuring diaphragm; 12, photomultiplyer tube.

epithelial cells, was collected by the objective lens. The loops of proximal tubules in frog kidney are situated near to the dorsal surface, forming nearly one layer. As we focused the microscope on the center of the tubule, the depth of the fluorescent volume was equal to the thickness of the tubule. Special controls were performed to make sure that there was no redistribution of the fluorescent dye after the kidney was taken out and rinsed with Ringer. As the measurements take about 3–4 min, we immersed the kidney for 10 min into the Ringer free of dye and measured the fluorescein content of tubules anew. No noticeable changes were detected. This is due to the fact that we deal with an undamaged organ and not with slices or isolated tubules.

As a rule we focused the microscope successively on 40 different tubules in the same kidney and repeated the measurements on 3–5 frogs. The control kidneys were measured in the same way. A series of 120–200 measurements was submitted to usual statistical computation. All straight lines on the plot were drawn by the least square method and correlation coefficients were always estimated. In the range of concentrations of fluorescein studied, its fluorescence is proportional to concentration [12]. The same is true for the intensity of fluorescence measured by our device, because all other parameters, including the geometry of the fluorescent object (depth and area of the observation field), are constant.

Some of our experiments were made for comparison on artificial membrane models. We used bimolecular lipid films, formed in a teflon cell. The films were prepared and studied according to the procedure of Mueller et al. [13].

RESULTS AND DISCUSSION

First of all the fluorescein content of a proximal tubule was measured as function of time (Fig. 2). We see that it increases in a linear way (the correlation coefficient with a straight line is 0.99). A similar dependence was found at higher fluorescein concentrations (up to 10^{-4} M). Hence we conclude that the rate of transport is constant during the time of incubation (20 min). The dependence of the trans-

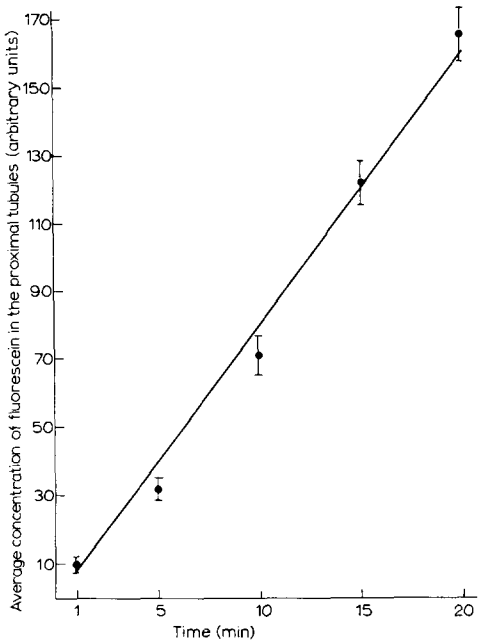

Fig. 2. Increase of concentration of fluorescein inside the proximal tubules of frog kidney as a function of time of incubation. Concentration of fluorescein in the medium is $3 \cdot 10^{-5}$ M. Each point is the mean value of 120–200 measurements. The vertical lines show the 95% confidence limits for each value.

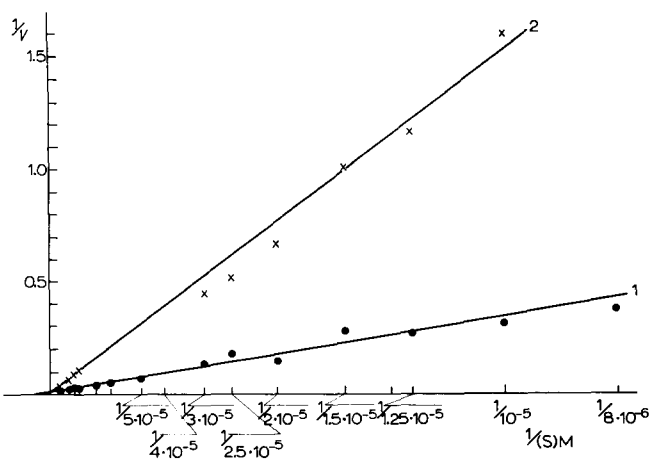

Fig. 3. Dependence of the rate of transport of fluorescein into the tubules on its concentration in the medium, plotted in reverse values, according to Lineweaver-Burk. (1) In the case of pure fluorescein in the medium; (2) in the presence of a competing agent, *p*-aminohippuric acid at a concentration 10^{-2} M.

port rate on substrate concentration conforms to a typical Michaelis-Menten curve. It is shown as a Lineweaver-Burk plot in Fig. 3. The experimental points fall on a straight line (correlation coefficient 0.98). Visual control shows that fluorescein is concentrated mostly inside the tubules. Its fluorescence in the cytoplasm is much

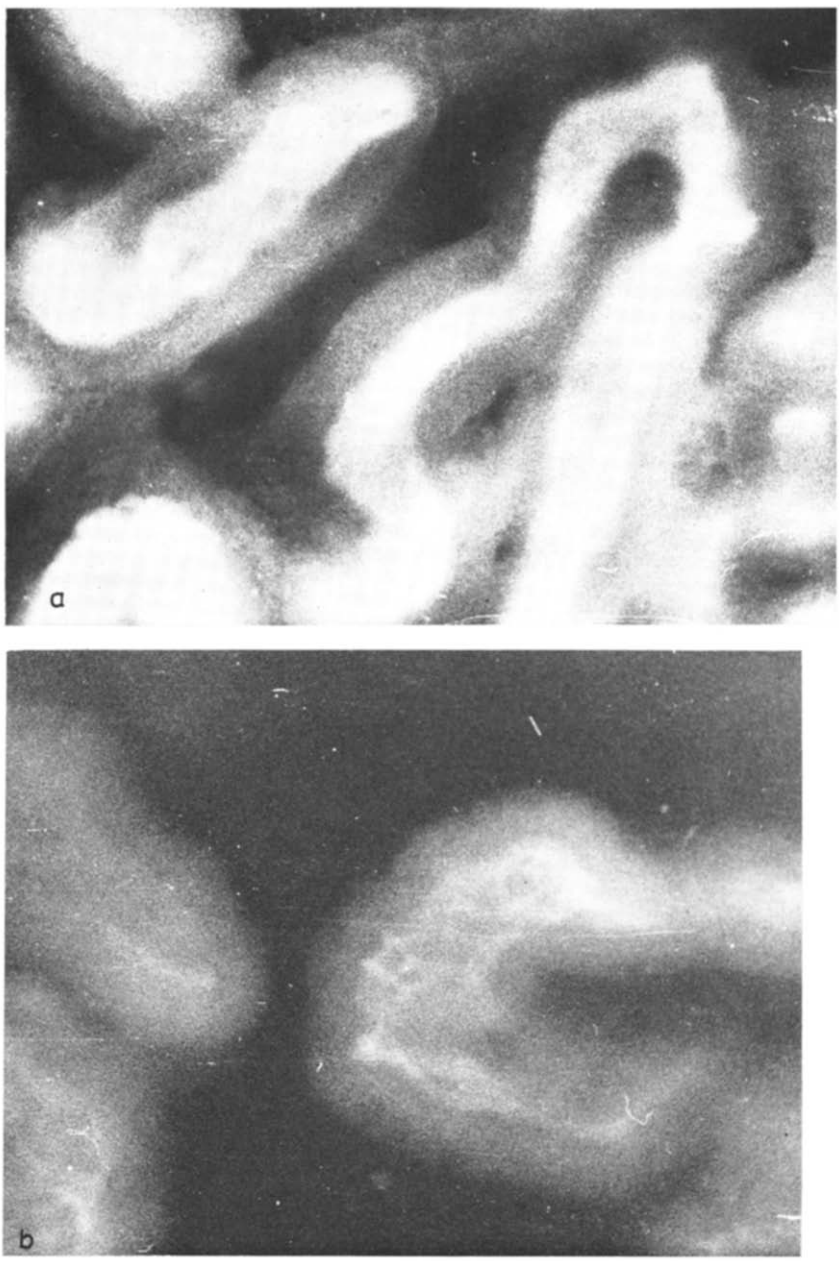

Fig. 4. Photograph of a fluorescent proximal tubule of frog kidney (a) in fluorescein solution and (b) in the presence of 10^{-2} M *p*-aminohippuric acid.

weaker (Fig. 4a). If another organic acid (*p*-aminohippuric) is added to the solution, the fluorescein transport is inhibited, but the initial rate of transport is restored by adding an excess of fluorescein. This shows that the inhibition of transport is competitive. To prove this fact in a quantitative way a Lineweaver-Burk plot was made for the transport rate as function of substrate concentration in the presence of *p*-aminohippuric acid. Both straight lines intersect the ordinate axis in the same point, as is usual in the case of competitive inhibition (Fig. 3). Visual control (Fig. 4b) shows that in this case the basal and apical membranes behave differently. The transport of fluorescein across the apical membrane is strongly inhibited in the presence of *p*-aminohippuric acid but through the basal membrane is mainly preserved. Therefore, in this case, the fluorescein content in the cytoplasm of the epithelial cells increases considerably. This property of effecting active transport across both the basal and the apical membrane is specific for frog kidney [9, 3, 14, 15]. In the kidneys of rat or rabbit, transport is active only in the basal membrane [16]. Therefore frog kidney is very advantageous for this study. We verified especially that the action of *p*-aminohippuric acid on the transport of fluorescein is absolutely reversible. Because of the different behaviour of the two membranes the kinetics of transport of fluorescein in the presence of *p*-aminohippuric acid ceases to be linear. We see from Fig. 5 that the rate of transport is preserved for about 5 min and then begins to decrease. The rates plotted on Fig. 3 are averages for 15 min. The dependence of the initial transport rate of fluorescein on the concentration of *p*-aminohippuric acid is hyperbolic (Fig. 6). Visual control confirms that at higher concentrations of *p*-aminohippuric acid the dye

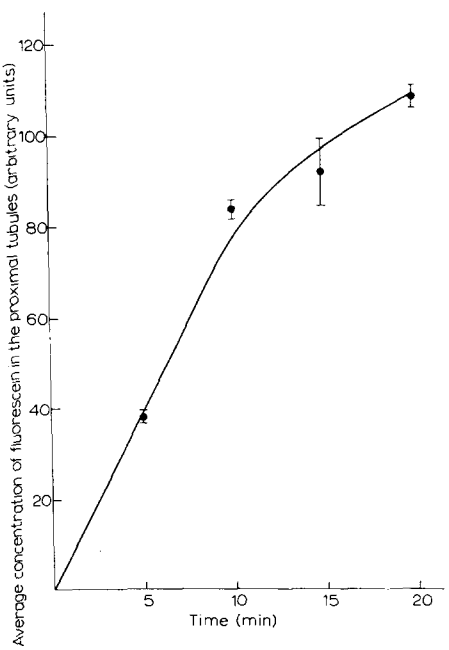

Fig. 5. Average concentration of fluorescein in the proximal tubules in the presence of *p*-aminohippuric acid after different incubation times. The medium content of fluorescein is 10^{-4} M and of *p*-aminohippuric acid 10^{-2} M. All other notations are as in Fig. 2.

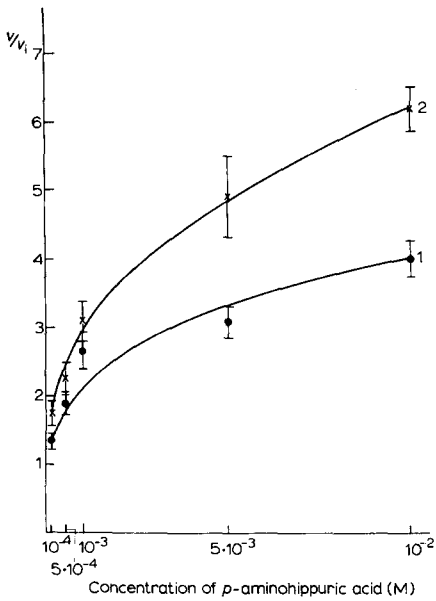

Fig. 6. Transport of fluorescein into the proximal tubules as a function of the concentration of *p*-aminohippuric acid in the medium. The ordinate shows the ratio of the transport rate without inhibition (v) to the rate in the presence of the inhibitor (v_1). Concentration of fluorescein (1) -10^{-4} M, (2) 10^{-5} M. Time of incubation 15 min. All other notations as in Fig. 2.

is accumulated much more strongly in the cytoplasm of epithelial cells. Its transport into the lumen of the tubule is partly or totally suppressed. Summing up, we see that the fluorescein transport from the outer medium into the lumen of the tubule is performed by a two-step mechanism.

Some experiments were specifically aimed at the molecular mechanism of active transport. First of all it was necessary to establish whether fluorescein is able to diffuse through a lipid barrier. For this purpose we used an artificial lipid membrane of the Mueller-Rudin type. It was found that the lipid (lecithin with octane) membrane is impermeable to fluorescein. No transport could be detected if a potential difference of 0.1 V was applied to the membrane in either direction. Therefore we are forced to admit that the biological membrane must contain a special substance (a ionophore) for the transport of organic acids through the lipid barrier. In this case the applicability of Michaelis-Menten equation and the competition of two different organic acids for transport becomes quite natural.

A second important point is the problem of energy source. It is widely accepted that there exists an $(Na^+ + K^+)$-dependent ATPase in the renal membrane and that it is acting as a sodium pump [5, 6]. In an earlier paper, it was shown that fluorescein transport is really coupled to Na^+ pumping from the tubules [9].

The energy source can be identified by means of specific inhibitors acting on the ATPase (ouabain or strophantin K). We used strophantin K as specific poison and found (Fig. 7) that it acts as an inhibitor of fluorescein transport. Visual control shows that transport into both the tubule and the cytoplasm of epithelial cells are

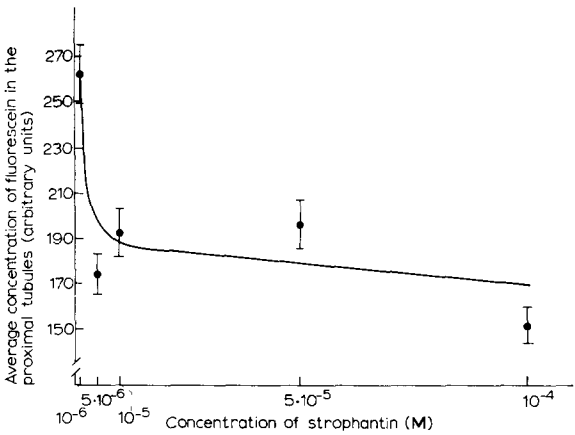

Fig. 7. Average concentration of fluorescein in proximal tubules at different concentrations of strophantin K in the incubation medium. Concentration of fluorescein in the medium $5 \cdot 10^{-5}$ M. Time of incubation 15 min. All other notations as in Fig. 2.

reduced. These facts confirm the coupling action of the enzyme, which establishes a sodium gradient in the membrane and induces the transport of organic acids.

The enzymatic nature of the transport phenomenon is emphasized by the temperature dependence of the transport rate of fluorescein (Fig. 8). The corresponding curve has a maximum at 18 °C and is quite typical of enzyme kinetics in case of cold-blooded animals. The Q_{10} value below the maximum is 1.36.

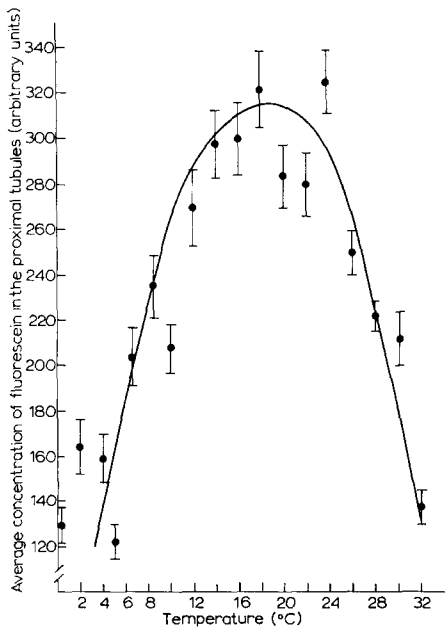

Fig. 8. Average concentration of fluorescein in proximal tubules as a function of temperature. Concentration of fluorescein 10^{-4} M, incubation time 15 min. All other notations as in Fig. 2.

Sodium ions are indispensable for the active transport of acids into the proximal tubules of the kidney. If an equimolar quantity of choline chloride is substituted for NaCl in the medium, the transport rate of fluorescein is only 41 % of the control (85.8±4.5 relative units in choline chloride versus 205.1±10.8 in NaCl medium). In this case the transport of fluorescein becomes passive and ceases when the concentrations inside and outside the tubules are equilibrated. According to the calculations of Hoshi and Hayashi [5] the transporting ionophore in the membrane of proximal tubules forms a complex with one organic anion and two sodium cations. The complex is therefore positively charged. The fact that the transport of acid into the tubule is coupled with the pumping of Na^+ from its lumen and requires the presence of Na^+ in the medium can be regarded as evidence of the validity of Mitchell's theory in this particular case. In the absence of Na^+, only passive diffusion of fluorescein via the ionophore remains.

If these ideas are correct we can predict that any factor able to discharge the membrane must reveal an inhibitory effect on the transport of fluorescein. It is well known that valinomycin, if introduced into the membrane, activates a passive flow of K^+ downfield and therefore discharges the membrane. This was shown for isolated mitochondria [17, 18] as well as for somatic cells [19, 20]. The introduction of valinomycin into the incubation medium of mitochondria induces at low concentrations (10^{-7}–$5 \cdot 10^{-7}$ M) a flow of K^+ into the mitochondria, but at higher concentration the escape of K^+ from mitochondria [17]. In our experiments we measured the transport of fluorescein in the presence of valinomycin (Fig. 9). The curve obtained is almost a precise mirror image of the dependence of K^+ transport into mitochondria on valinomycin concentration. The transport rate of fluorescein falls, passes through a minimum and returns to the initial value at higher concentrations. This response of the transport system of organic acids to valinomycin correlates with the discharge of the membrane by K^+ flux and is another argument in favor of the Mitchell's mechanism of active transport.

Now we turn to the description of a quite new phenomenon found during the

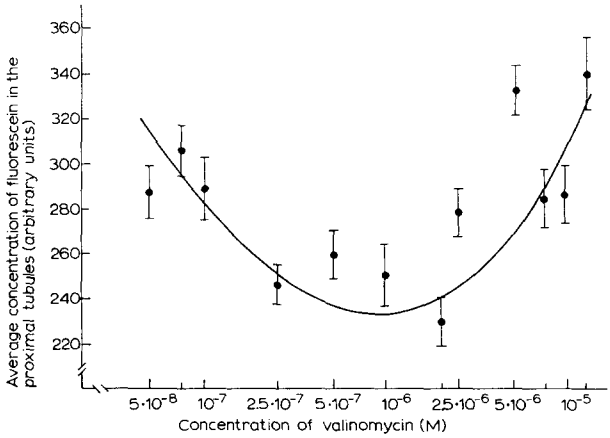

Fig. 9. Average concentration of fluorescein in proximal tubules as a function of concentration of valinomycin in the incubation medium. Concentration of fluorescein 10^{-4} M. Incubation time 15 min. All other notations as in Fig. 2.

study of fluorescein transport across the kidney membrane. The experiment was performed because it was expected that the lipid membrane is structurally a liquid-crystal (smectic phase). If so, it must consist of polymolecular domains. The ionophores, which conduct the ionic current through the membrane, are expected to penetrate the lipid layer and build channels for the ionic current. For some model substances with the properties of ionophores, such channels were established [21]. It was expected that the liquid-crystalline domains are capable of orientation in electric and magnetic fields because of electric or magnetic anisotropy of the molecules in question. The effect of magnetic fields is particularly interesting, because their action on single molecules is insignificant. It can be estimated by the criterion $\mu H/kT$, the ratio of the magnetic energy of a molecule (μ is the induced magnetic moment in case of diamagnetic substance, H is the fieldstrength) to the thermal energy (k is the Boltzmann constant, T is the absolute temperature). For a diamagnetic substance and a field $H = 10^4$ oersted, this ratio is of the order 10^{-5}–10^{-6}. Therefore no measurable orientation of a molecule in a magnetic field is ever possible. But the situation changes in case of diamagnetic domains in liquid crystals. As is well known, these are readily oriented in magnetic fields [22]. Therefore we expected a marked influence of a magnetic field on the active transport of fluorescein through the membrane. The sign of the expected effect could not be predicted, because it depends on such properties as magnetic anisotropy of molecules, which are unknown. But we succeeded in finding the effect, and it appeared to be considerable and easy to measure, when we used magnetic fields up to 28 000 oersted [11].

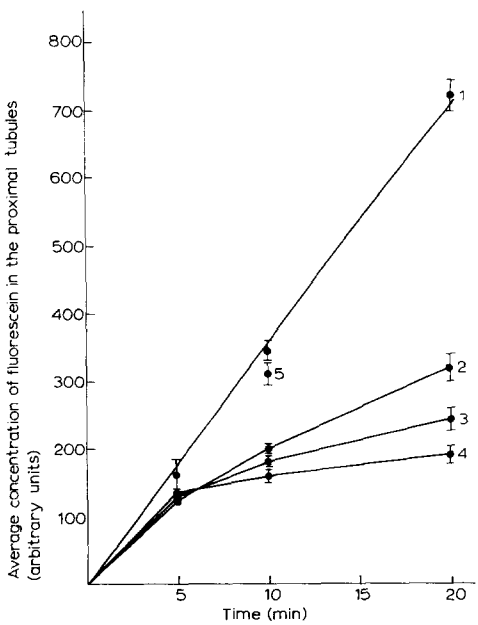

Fig. 10. Average concentration of fluorescein in proximal tubules as a function of time after incubation in magnetic fields. (1) Incubation without field (control). (2) Field strength 10 000 oersted, (3) 20 000 oersted, (4) 28 000 oersted. (5) Incubation during 10 min at 28 000 oersted and then 10 min without field. All other notations as in Fig. 2.

When incubation of the kidney in fluorescein solutions was effected in a constant magnetic field, after an initial period of few minutes the transport rate dropped (Fig. 10). The experiment is quite simple. The vessel with the kidney immersed into the fluorescein solution is inserted between the poles of an electromagnet and kept there for the time needed. Afterwards the kidney is rinsed and the content of fluorescein in the tubules measured in the usual manner. A second kidney is used as a control and immersed into the same solution outside the field. There are no physicochemical reasons for any action of a magnetic field on metabolic processes, enzyme kinetics and so on. The only known and logically feasible possibility is the orientation of liquid crystalline domains [11]. At the highest field strength active transport stopped practically entirely. When the field was switched off the active transport resumed at the same rate. The phenomenon of transport inhibition was totally reversible. Visual control showed that the transport of the dye into the lumen of tubules is inhibited and that the degree of inhibition depends on the orientation of tubules relative to the magnetic field. In some tubules the transport is inhibited at lower fields. Some others with a less advantageous orientation preserve the ability to concentrate fluorescein till higher fields. Of course the curve of Fig. 10 demonstrates an averaged effect. The fact that magnetic field influences the active transport so strongly is, to our understanding, a direct evidence of the liquid-crystalline structure of biological membranes and demonstrates the crucial importance of this structure for its function. Of course we must take into consideration that the field is acting on all membranes of cells (plasma membranes, mitochondria and so on). The effect of inhibition of fluorescein transport across the apical membrane that we observe may reflect the statistical sum of a multitude of effects primarily induced by the magnetic field in all liquid-crystalline membranes of cells.

CONCLUSION

The experiments described show that the active transport of a marker acid, fluorescein, into the proximal tubules of frog kidney is effected by means of a special carrier, which forms a positively charged complex with the organic anion and sodium. The complex moves downfield under the action of an electrochemical gradient, produced by the $(Na^+ + K^+)$-dependent ATPase. The lipid layer of the membrane reveals a liquid-crystalline structure. Hence the orientation of lipid domains in magnetic fields has a strong influence on the transport rate.

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