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ON THE ACTIVE TRANSPORT OF ORGANIC ACIDS (FLUORESCEIN) IN THE CHOROID PLEXUS OF THE RABBIT

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Summary

The kinetics of active transport of an organic acid (fluorescein) through the membranes of the choroid plexus from the lateral ventricules of the brain of rabbit was studied both morphologically and functionally. It was shown that fluorescein is actively translocated through the apical and basal membrane of the epithelium and is accumulated in blood capillaries at a concentration exceeding one order of magnitude that in the incubation medium. The kinetic curves displaying saturation and the demonstration of inhibition by other acids shows that a specific carrier is involved in the transfer across the membrane. The active transport of fluorescein at 20°C was found to be sodium independent. Total exclusion of sodium from the incubation medium does not change the Michaelis constant (K_m) and maximal velocity (V). The active transport depends on the operation of ($Na^+ + K^+$)-ATPase as energy source but obviously no specific complexes with the participation of sodium are involved.

Introduction

At present the most general concept concerning the mechanism of transport across cellular membranes is the gradient hypothesis [1—5]. According to this an electrochemical gradient of Na^+ created by sodium pumps is the motive force for transfer of organic solutes across plasma membranes of eukaryotic cells. Organic cations are translocated with special carriers by the action of the electric field in the membranes. Neutral molecules and organic anions form a transport complex containing besides a carrier one (neutral molecules) or two (organic anions) sodium ions, so that the complex becomes electropositive. Thus, the transport of neutral molecules and organic anions is mostly Na^+ dependent.

An alternative hypothesis has been introduced by Csaky [6] and Kimmich [7]. It assumes the transport of organic solutes to be the result of the phosphorylation of the carrier protein by the $(Na^+ + K^+)$-ATPase. This hypothesis was reported to obtain experimental support [8].

It is not unlikely that both hypotheses are to some extent true. To check this possibility an appropriate model is needed. The uptake of weak organic acids in the choroid plexus of lateral brain ventricules seems to be an interesting model for this purpose. In the plexus a special carrier-mediated system is known to transport organic acids [9—17]. However, the localization and mechanisms of operation of this system has to be studied more thoroughly. In the present paper the dependence of the system on ionic environment and an $(Na^+ + K^+)$-ATPase operation was studied. Earlier, a method of visualization of active uptake in an intact organ was developed. It uses fluorimetric measurements of the concentration of a fluorescent substrate (fluorescein) in the cell and capillary interiors by means of a microfluorimeter. The method was used with success in the case of proximal tubules of kidney [18—20] and for the study of transport in choroid plexus [21]. It enabled us to work with undamaged organs and to distinguish between the flow across the apical and basal membrane of the cell.

Materials and Methods

All experiments were performed with 2—3 kg 'Chinchilla' male rabbits. The animals were killed by air emboly, both choroid plexuses were excised from lateral ventricules of the brain and incubated in isotonic salt solution with fluorescein at room temperature (20°C) or at 37°C with aeration by pure oxygen. As salt medium we used a Krebs-Ringer phosphate buffer containing (in mM): 111 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 2.4 $MgSO_4$, 1.2 KH_2PO_4, 3.6 $NaHCO_3$, 4.8 Na_2HPO_4, 2.1 NaH_2PO_4, 11.7 glucose, pH 7.4—7.5. In some experiments we used a Hanks solution containing (in mM): 136.8 NaCl, 5.4 KCl, 1.25 $CaCl_2$, 0.4 $MgSO_4$, 0.5 $MgCl_2$, 0.34 Na_2HPO_4, 0.45 KH_2PO_4, 5.5 glucose, 4.1 $NaHCO_3$, pH 7.4—7.5. The pH of the Krebs-Ringer and Hanks solution was controlled and remained constant during a whole experiment (in the range of 0.1). As a medium with low Ca^{2+} concentration we used Hanks solution but with only 0.25 mM $CaCl_2$. To keep the osmolarity constant the concentration of NaCl was correspondingly increased. The media devoid of sodium were of three types. A Hanks solution was prepared but with the substitution of NaCl by choline chloride, sucrose or LiCl. In all cases $NaHCO_3$ was substituted by $KHCO_3$. The osmolarity of the modified solutions was kept constant, pH 7.4—7.5. The medium devoid of potassium was prepared by substitution of potassium by sodium. To study the influence of pH we used Hanks solution with the exclusion of $NaHCO_3$. It contained a 0.01 molar phosphate buffer of pH 6.0, 7.0 or 8.0. The pH of the media was controlled and remained constant.

In some of the experiments the blood was washed off from the blood vessels. It was performed by a perfusion of the brain through both arteria carotis communis with the corresponding sodium-free solution at a pressure of 300 mm water. Controls were perfused in the same way by sodium-containing

solutions. The incubation of the plexus was carried out in the fluorescein solution for the desired time. The plexus was then rinsed in a similar solution but without fluorescein and stratified on a microscope object glass of a special non-fluorescent sort. The cover glass was of quartz. The intensity of fluorescence was measured in a microfluorimeter with a stabilized iodine lamp as light source and two exchangeable light probes [22]. We used a combination of excitation and barrier light filters which practically cut off all background and let through the fluorescent light (500—570 nm). The diameters of the light probes in the plane of the object were 4.5 and 27 μm. The fluorescent light from the choroid plexus was a measure of the concentration of fluorescein within the object. The electric current in the photomultiplayer tube was a measure of fluorescein concentration in arbitrary units. The accuracy of microfluorimetric measurements was controlled by registering the luminescence of a standard uranium glass. Each choroid plexus was moved under the microscope and the intensity of fluorescence was measured at 30—40 different points. The duration of measurements on a chosen plexus was less than 3 min. The autoluminescence of the choroid plexus was not detectable under our conditions. Therefore the luminescence measured can be attributed only to fluorescein accumulation in the tissue. All measurements were repeated on 3—5 organs from different animals. All 90—200 experimental figures were averaged in the customary manner and 95% confidence limits were calculated for every point on the graphs. For the estimation of apparent K_m and V a linear Lineweaver-Burk equation was composed by the least squares method and computed [19]. Besides quantitative measurements, we studied the morphology of active transport, making photographs of the corresponding pictures.

Results

1. Morphology of active transport in the choroid plexus

The microscopic investigation of the plexus after incubation in Krebs-Ringer solution containing fluorescein shows that the dye is accumulated in the folds (villi) of the plexus. In an optical projection these folds are seen as tubes of 30—40 μm diameter. In the middle of a fold a capillary blood vessel of 7—10 μm diameter is situated. The apical membrane of a layer of neuroepithelial cells in the folds is turned towards the incubation medium, the basal membrane is turned towards the capillary wall (Fig. 1, a and b). Fluorescein accumulated in the cells is distributed evenly (with the exception of the nuclear area). Fluorescein accumulated in the lumen of blood vessels is dissolved in the blood plasma. The concentration of fluorescein in the lumen of blood vessels is higher than that in the neuroepithelium (Fig. 1a). The erythrocytes remain unstained (Fig. 1c). The layer of neuroepithelial cells with the capillary vessels adhering to the basal membrane can be regarded as a fundamental structure unit effecting active transport.

In the middle part of the choroid plexus the folds look like a system of tightly packed parallel tubes (Fig. 1a); at the edge of the plexus the folds are located more irregularly (Fig. 1b) and less tightly (Fig. 1c).

2. Kinetics of fluorescein transport through the cells of choroid plexus

At first the concentration of fluorescein accumulated in the folds of plexus

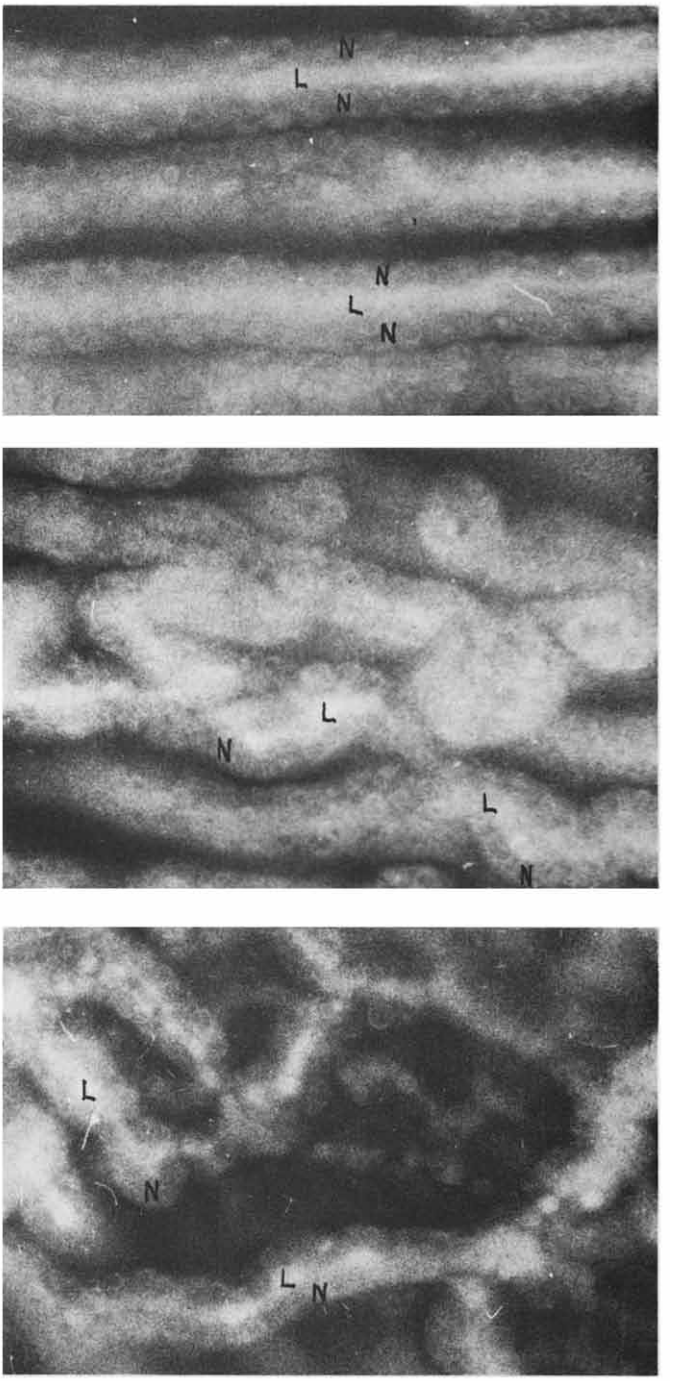

Fig. 1. Morphology of the active transport of weak organic acid (fluorescein) in choroid plexus. a, parallel tight localization of the folds (villi) in the middle part of choroid plexus; b, irregular tight localization of the folds near the caudal part of plexus; c, irregular and rare localization of the folds in the caudal part of plexus. The fluorescein of the bath medium is practically invisible. Neuroepithelium (N)-forming folds contain visible amounts of fluorescein. The concentration of the dye in the lumen (L) of blood vessels inside the folds is much higher. Fluorescent microscopy. Magnification 300X.

was investigated as a function of incubation time. As seen in Fig. 2 the concentration in the folds increases linearly with time, i.e. the uptake rate is constant at least during 0.5 h. It was shown earlier [23] that the intensity of fluorescence in thin layers of fluorescein solution is directly proportional to its concentration in wide ranges (10^{-6}—$5 \cdot 10^{-3}$ M). However, binding of fluorescein in the cytoplasm is quite possible. It results in a 2-fold decrease of the quantum yield of luminescence [23]. The preceding perfusion of the capillary vessels by salt solution does not change the luminescence intensity of fluorescein accumulated inside the capillaries, hence it can be concluded that fluorescein is not absorbed on plasma proteins to a measureable degree.

For the determination of the absolute fluorescein concentration the data on the fluorescein uptake after incubation of the choroid plexus in anoxic conditions (bubbling of pure argon) during 0.5 h, a time shown to be sufficient for equilibration, were used (Fig. 2). The fact that in such conditions there is no active transport is easily seen because there is no difference between the fluorescence of the capillary vessels and the cytoplasm. Therefore we can conclude that the fluorescein concentration inside the plexus is equal to the external one.

Now we can estimate the accumulation of fluorescein in the plexus in absolute units. After 25 min of incubation at 20°C in a Krebs-Ringer phosphate buffer containing $5 \cdot 10^{-5}$ M fluorescein the concentration of the dye in the cytoplasm is 2.5 times higher than that in the bath medium. The concentration inside the blood vessels is 12 times higher than that in the medium. These measurements were made by means of small light probes of the fluorimeter (4.5 μm in diameter) which enabled us to measure the luminescence intensity inside a single capillary. The average concentration of fluorescein in the epithelial fold

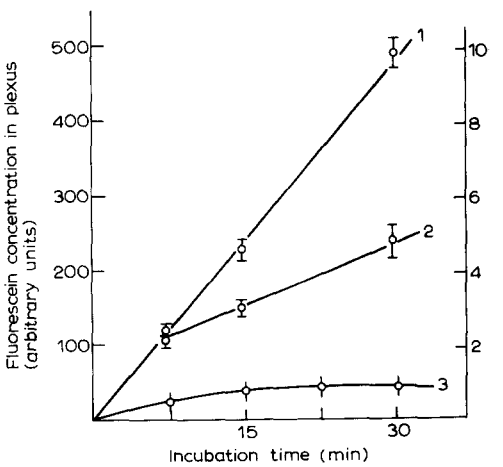

Fig. 2. Increase of the average fluorescein concentration in the folds ot the plexus as a function of time. The bath medium is saturated (1) by pure oxygen, (2) by air, (3) by argon. The fluorescein concentration in the bath medium is $5 \cdot 10^{-5}$ M, temperature 20°C. Each point on the curves is the average of 90—200 measurements on 3—5 rabbits. The vertical lines show the 95% confidence limits. Scale on the right gives the ratio of the fluorescein concentration in the plexus to the concentration in the bath medium (*T/M* ratio).

(measured with 27 μm diameter light probe) is 8.5 times higher than that in the solution. If the concentration of oxygen in the solution is lowered (the medium was equilibrated with air instead of oxygen) the uptake of fluorescein is reduced (Fig. 2).

The uptake in both normal salt media (Krebs-Ringer or Hanks) was absolutely identical. The concentration of fluorescein in the medium increasing from $5 \cdot 10^{-6}$ M to $5 \cdot 10^{-4}$ M the uptake rate at 20°C rose in a hyperbolic way and tended toward a plateau (Fig. 3). This curve conforms satisfactorily to the Michaelis-Menten equation. The correlation coefficient with the linear Lineweaver-Burk plot is 0.993. The average K_m is $4 \cdot 10^{-5}$ M.

p-Aminohippuric acid was tried in its capacity as a well known substrate for the carrier-mediated uptake of weak organic acids in the choroid plexus [9,11, 12,15,17] simultaneously with fluorescein uptake. It inhibited the uptake at 20°C (the ratio of concentrations *p*-aminohippuric acid : fluorescein was equal to 200) by 40% after 15 min incubation. The cardiac glycoside strophantin K ($5 \cdot 10^{-5}$ M) reduced fluorescein uptake (the dye concentration in the bath medium was $5 \cdot 10^{-5}$ M) at 20°C by 15% and at 37°C by 50% after 30 min incubation. It should be noted that the rate of fluorescein uptake at 37°C in the control (free of strophantin) was 1.9 times greater than that at 20°C. If the concentration of potassium ions is increased to 60 mM, the action of strophantin K on the transport rate is totally levelled. This can be regarded as proof that strophantin and potassium are competing for the same binding sites [26].

When the pH of the bath medium at 20°C was changed from 6.0 to 8.0, the amount of accumulated fluorescein increases by 60%. Visual control shows that the distribution of the dye alters. As seen on Fig. 4a at pH 6.0 fluorescein is mainly accumulated in the cytoplasm of epithelial cells but very slightly in the

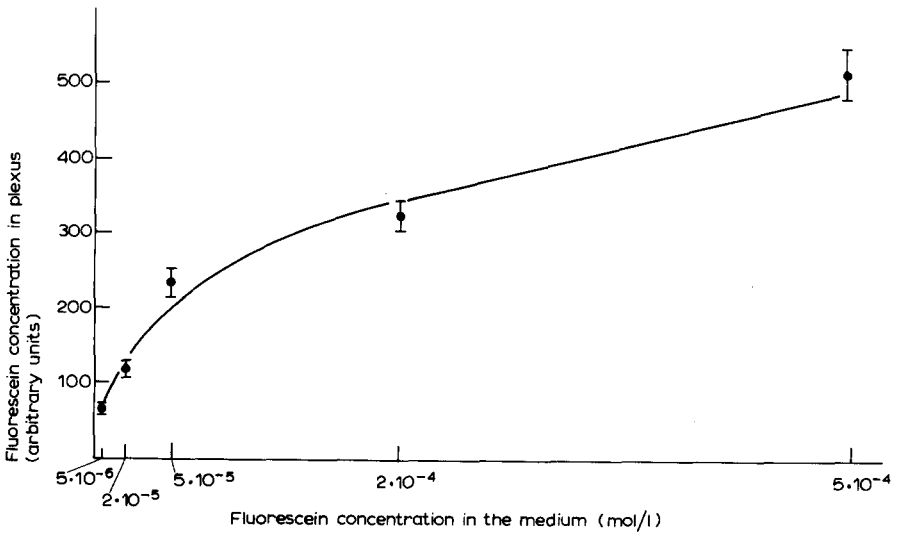

Fig. 3. Dependence of the uptake of fluorescein into the folds of plexus on the concentration of the dye in the medium. Temperature, 20°C; incubation time, 15 min. All other notations as Fig. 2.

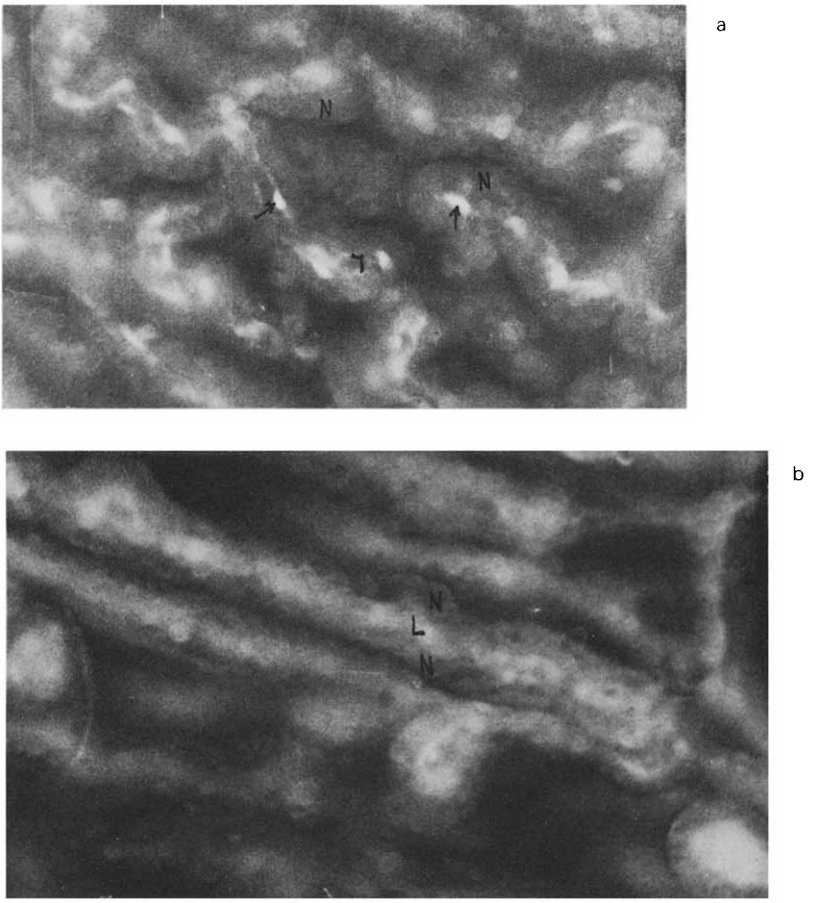

Fig. 4. Morphology of the transport of fluorescein in choroid plexus at different pH values of bath medium at 20°C. a, pH 6: the accumulation of the dye in the lumen of vessels is inhibited. The luminescence of the endothelial cells is rather bright (arrow). b, pH 8: the accumulation in the blood vessels is very pronounced. All other notations as Fig. 1. Fluorescent microscopy. Magnification 300X.

blood vessels. At pH 7.0, and particularly at pH 8.0, the accumulation in the blood vessels is very pronounced (Fig. 4b). Hence, the diminution of fluorescein concentration at pH 6.0 is due to decreasing fluorescein transfer from the cells into blood capillaries.

3. Action of cations on fluorescein transport

A 10-fold decrease (to 0.25 mM) of the concentration of calcium in the bath medium reduces the transport rate at 20°C by 35%. The rate of the dye uptake remains constant during the incubation under such conditions (not shown). Strophantin K ($5 \cdot 10^{-5}$ M) when added to the bath medium with low calcium inhibits the transport rate at 37°C by 57%. Visual control shows that low calcium concentration does not affect in any way the morphology of transport, but some adsorption of the dye on the erythrocyte surface is eventually visible showing that they become labile and easily damaged.

We studied the uptake rate of fluorescein in the case when sodium chloride

in the bath medium was substituted by choline chloride, lithium chloride or sucrose. To remove sodium ions from the blood vessels the choroid plexus was washed by a sodium-free solution by means of perfusion through both arteria carotis communis. As seen from Fig. 5 the absence of Na in the medium has no influence on the active transport. Both kinetic constants (apparent K_m and V) remain the same. They coincide within the range of error with the kinetic constants measured earlier without perfusion of the organ. Therefore we are sure that the procedure of perfusion and the complete absence of sodium in the bath medium has no meaning for the transport.

We studied the action of a sodium-free solution in the case of low calcium. At first a considerable increase of net accumulation of the dye was obtained, but it was found to be an artefact. Visual control showed that under such conditions the accumulation of fluorescein inside the blood capillaries is increased and the erythrocytes are highly stained, obviously because of strong damage. After preliminary washing off of blood vessels by perfusion we found that there is no difference between the transport of fluorescein in the case of sodium-containing or sodium-free medium.

When potassium was eliminated from the bath medium (it was substituted by sodium) the transport rate was inhibited by 58%. Preincubation of choroid plexus for 15 min in potassium-free solution resulted in an inhibition of fluorescein uptake in K^+-free bath media by 73% as compared to the corresponding control. On the other hand, if we increase the concentration of K^+ in the bath medium an increase of transport rate results till a concentration of 60 mM (Fig. 6).

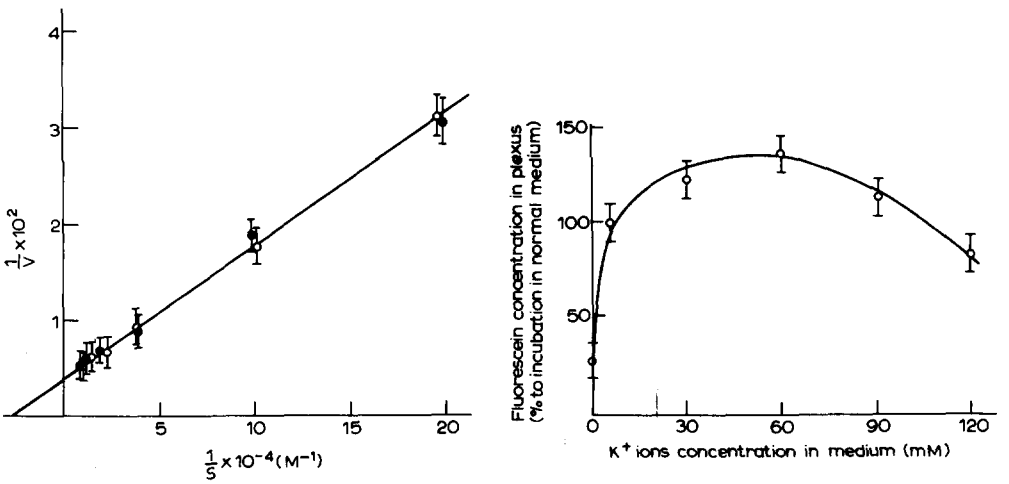

Fig. 5. Lineweaver-Burk plot for active transport of fluorescein from a sodium-containing (open circles) or sodium-free (filled circles) bath medium at 20°C. Incubation time, 15 min; apparent K_m $3.7 \cdot 10^{-5}$ M for both cases. All other notations as Fig. 2.

Fig. 6. Average content of fluorescein in the folds of the choroid plexus after 15 min incubation at different K^+ concentrations in the medium. Concentration of fluorescein in the medium, 10^{-4} M; temperature, 20°C; incubation time, 15 min.

Discussion

Visual observation and microfluorimetry of the active transport into the cytoplasm of neuroepithelial cells and into the lumen of capillary vessels showed that there exist two stages of fluorescein transport: one from the external bath medium into the cytoplasm across the apical membrane of cells, the second from the cytoplasm into the capillary vessels across the basal membrane. Both increase the concentration of the dye, the former 2.5 times, the latter 12 times comparing with the concentration in bath solution. If we used a 27 μm light probe, the intensity was averaged, but it is evident that about one third of the measured area is occupied by blood vessels and the contribution of the latter to the total fluorescence is not less than 70%. In most of the quantitative measurements just this diaphragm was used.

The transport systems of the apical and basal membranes behave in a different way if the pH is changed. The basal membrane is more sensitive to low pH. Rall and Sheldon [9] studied the transport of chlorphenol red and phenol red into the choroid plexus of the dogfish and observed in the microscope the accumulation of the dyes in the blood vessels but did not notice any change in the cytoplasm. We find that concentration in the blood vessels is much more pronounced but we see the increase also in the cytoplasm. Probably the observation of fluorescence has the advantage of much superior sensitivity if compared with absorbance measurements used by the authors quoted. The removal of blood from capillary vessels by perfusion with an ordinary salt solution has no influence on the accumulation of fluorescein in the blood vessels. The kinetics of active transport of fluorescein into the choroid plexus is characterized by a function, approximated with good precision by the Michaelis-Menten equation. Introduction of a second acid (*p*-aminohippuric) into the bath medium inhibits the transport rate in a competitive way. Both facts are direct indications of active transport effected with participation of a specific carrier.

Since fluorescein transport depends strongly on the O_2 content and is totally inhibited under anoxic conditions, we may assume that oxidative phosphorylation is the only energy source. Such a conclusion is in general agreement with published data concerning inhibition of organic acid transport in the choroid plexus by anoxia, cyanide and 2,4-dinitrophenol [9—12,14,15,17].

The rate of fluorescein transport in the rabbit choroid plexus depends on ionic environment. A decrease (or omission) of Ca^{2+} from the bath medium results in an inhibition of transport of both fluorescein and other organic acids [9,10]. This phenomenon is probably connected with the stabilizing action of Ca^{2+} on the structure of biological membranes [24].

The complete omission of Na^+ from the bath medium did not influence the active transport of fluorescein at 20°C. Hence, under such conditions fluorescein transport is not sodium-dependent. An analogous system of transport in renal proximal tubules is known to be sodium-dependent [19,25].

Complete omission of potassium from the bath medium resulted in a twofold reduction of fluorescein uptake at 20°C. After preincubation in K^+-free solution the uptake of fluorescein was inhibited to a greater extent. A similar phenomenon was observed by Rall and Sheldon [9]. An increase of K^+ concen-

tration stimulates fluorescein transport. K^+ in the bath medium is indispensable for the operation of $(Na^+ + K^+)$-ATPase. A high concentration of this cation in the medium interferes with the inhibitory action of cardiac glycosides on the enzyme, as was shown earlier [26]. This fact may account for the disappearance of the inhibitory action of strophantin on fluorescein uptake at 60 mM of K^+.

The inhibition of uphill uptake of fluorescein in the choroid plexus by strophantin K at both 20 and 37°C is not complete. The same is true for strophantin G inhibition of $(Na^+ + K^+)$-ATPase activity [26].

The cardiac glycosides are specific poisons of $(Na^+ + K^+)$-dependent ATPase. The enzyme requires K^+ outside the cell and Na^+ inside. Obviously these requirements are fulfilled. But if the Mitchell mechanism is operational in our case, there must be a different cation, not sodium responsible for the movement of organic acids into the choroid plexus because the membrane is negatively charged inside and the electric field is a barrier against the translocation of anions. The same is true for a gradient of H^+ in the membrane. An uptake of organic acid by a pH gradient on the membrane would require a more alkaline medium inside the cell, but this is not in accord with the fact that the fluorescein uptake is stimulated by an increase of pH outside the choroid plexus.

Possibly the case of active transport in the choroid plexus is independent of an ionic gradient in the membrane. A final solution for the mechanism of active transport requires more experimental work.

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