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# Different Sodium Chloride Cotransport Systems in the Apical Membrane of Rabbit Gallbladder Epithelial Cells

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**Summary.** The kinetics of $Cl^-$ influx from the lumen to the cell and the paracellular pathway was examined in isolated rabbit gallbladder by measuring $^{36}Cl$ uptake (45 s) and by correcting it for the extracellular space with $^3H$-sucrose. The paracellular fraction of the influx was studied by incubating the tissue in $Na^+$-free saline or in solutions containing 25 mM $SCN^-$; the kinetics turned out to be hyperbolic. The cellular fraction of the influx comprised three components. The first was immediately $Na^+$-dependent and insensitive both to exogenous and endogenous cell bicarbonate; its sigmoidal kinetics revealed the presence of a carrier with three $Cl^-$ binding sites cooperating positively with one another, with strong interaction factors. The second cellular component was immediately $Na^+$-dependent and sensitive to endogenous cell bicarbonate; the kinetics was hyperbolic with a maximum at 20 mM $Cl^-$ concentration and a substrate inhibition from 20 to 130 mM; it was completely inhibited by $10^{-4}$ M acetazolamide. The third cellular component was slowly $Na^+$-dependent and slowly sensitive to exogenous bicarbonate; its kinetics was hyperbolic, without substrate inhibition in the tested $Cl^-$ concentration range. On this basis, the presence of three $Na^+-Cl^-$ cotransports is suggested: i) on a single carrier without any exchange with $H^+$ and $HCO_3^-$, ii) on a single carrier with an exchange with $H^+$ and $HCO_3^-$, and iii) on two separate carriers in exchange with $H^+$ and $HCO_3^-$.

**Key Words** gallbladder · $Na^+-Cl^-$ cotransport · $Cl^-/HCO_3^-$ exchange · $Na^+/H^+$ exchange · paracellular $Cl^-$ influx

## Introduction

In 1973 Nellans, Frizzell and Schultz demonstrated that $Cl^-$ entered the epithelial cells of rabbit ileum by a neutral cotransport with $Na^+$. A similar cotransport was found to be present in rabbit gallbladder (Cremaschi, Hénin & Ferroni, 1974; Cremaschi & Hénin, 1975; Frizzell, Dugas & Schultz, 1975) and in many other leaky epithelia (for a review, *see* Frizzell, Field & Schultz, 1979). The model proposed to explain these results was based on a single carrier able to transfer $Na^+$ and $Cl^-$ simultaneously, the former ion along its electrochemical potential gradient and the latter against it.

In 1976 Murer, Hopfer and Kinne and in 1977 Liedtke and Hopfer demonstrated for rat intestine a $Na^+-Cl^-$ cotransport which seemed to be due to a double exchange of $Na^+/H^+$ and $Cl^-/HCO_3^-$ (or $Cl^-/OH^-$ or possibly a symport of $H^+-Cl^-$) on two separate carriers. Sodium and chloride transepithelial transport, however, is also stimulated by $HCO_3^-$ present in the bathing fluid and in 1979 Cremaschi, Hénin and Meyer demonstrated that in gallbladder this stimulation is mainly exerted at the apical barrier on NaCl cotransport. They proposed two alternative models to explain how this interaction could take place: i) bicarbonate allosterically modifies the cotransport carrier by increasing its efficiency; ii) the cotransport carrier actually transfers $Na^+$ and $Cl^-$ in exchange with $H^+$ and $HCO_3^-$, respectively (endogenous bicarbonate being exchanged in the absence of the anion in the bathing fluids). Finally, in 1981 Heintze, Petersen and Wood and Petersen, Wood, Schulze and Heintze confirmed that exogenous bicarbonate mainly operated at the apical border and demonstrated that many weak acids exhibit similar effects, increasing their action as a function of their liposolubility. This makes the allosteric effect of bicarbonate unlikely and these authors therefore suggested the presence of a double exchange mechanism on two separate carriers following the model proposed for rat intestine; in principle, this double exchange should support only bicarbonate stimulation, but it could also account for the basal transport.

As a conclusion, so far three different models have been proposed to explain the mechanism of $Na^+-Cl^-$ cotransport: i) an actual cotransport on the same carrier, ii) a double exchange on the same carrier, and iii) a double exchange on separate carriers with only a functional and indirect coupling of $Na^+$ and $Cl^-$ mediated by $H^+$ extruded into the lumen.

In order to provide better insight on this subject, we have investigated the kinetics of $Cl^-$ entry through the apical membrane of rabbit gallbladder, where no $Cl^-$ conductance exists (Cremaschi & Hénin, 1975; Hénin & Cremaschi, 1975; Cremaschi & Meyer, 1982) and where the total $Cl^-$ influx is $Na^+$ dependent (Cremaschi & Hénin, 1975; Cremaschi et al., 1979) and inhibited by $SCN^-$ (Cremaschi et al., 1979).

## Materials and Methods

### *Gallbladder Preparation*

New Zealand rabbits were killed by a blow on the neck. Gallbladders were excised and washed free of bile with Krebs-Henseleit solution.

### *Measurement of $Cl^-$ Uptake*

Gallbladders, opened flat, were mounted, carefully avoiding any stretch, on a nylon mesh between two Lucite® chambers with the luminal surface facing upwards, exposed (0.38 $cm^2$) within the upper chamber, filled with 1 ml saline. After a preincubation period of 30 min in Krebs-Henseleit solution, the tissue was allowed to equilibrate for 45 min again in Krebs-Henseleit solution or in a bicarbonate-free saline. Then it was washed 5 times on the luminal side with the test solution (5 s) and finally exposed for 45 s to the same saline added with $^3H$-sucrose (10 μCi/ml) and with $^{36}Cl$ (4 μCi/ml). Variations in the preincubation on the luminal side will be described later. The influx (μeq $cm^{-2}$ $h^{-1}$) was constant for at least a 75 s exposure, so that by using a 45 s period, unidirectional influxes were measured (Cremaschi et al., 1979). Fluid stirring in the luminal solution was obtained by bubbling the appropriate gas mixture; conversely, the serosal chamber was continuously perfused with gassed saline moved by a peristaltic pump. At the end of the experiment the medium was withdrawn, the tissue squeezed with an ice-cold isotonic solution of mannitol and punched off, then processed as previously described (Cremaschi & Hénin, 1975). Radioactive material was purchased from the Radiochemical Centre, Amersham, Bucks U.K.

### *Salines*

Krebs-Henseleit solution contained (mM): $Na^+$ 143, $K^+$ 5.9, $Ca^{2+}$ 2.5, $Mg^{2+}$ 1.2, $Cl^-$ 127.7, $HCO_3^-$ 25, $SO_4^{2-}$ 1.2, $H_2PO_4^-$ 1.2; pH 7.4. Bicarbonate replacement was with $SO_4^{2-}$ and mannitol; $Cl^-$ was replaced by $SO_4^{2-}$ and mannitol or by ethanesulfonate as indicated; N-methyl-D-glucamine was substituted for $Na^+$; when $SCN^-$ was present, it was substituted for $Cl^-$ (in the solutions containing bicarbonate) or $SO_4^{2-}$ and mannitol (in the bicarbonate-free solutions)[1]. Salines were bubbled with 5% $CO_2$–95% $O_2$ when $HCO_3^-$ was present, with 100% $O_2$ in its absence. Incubation temperature was 27±1 °C.

## Results

### *$Cl^-$ Uptake in the Presence of Exogenous Bicarbonate*

Figure 1 reports the kinetics of $Cl^-$ influx obtained when 25 mM bicarbonate was present in the bathing solutions on both sides (kinetics *a* in the Figure). $Cl^-$ concentration was reduced in the lumen only during the measuring time (45 s) by substitution with $SO_4^{2-}$ and mannitol. With 127.7 mM luminal $Cl^-$ concentration the influx was 20.4 μeq

[1] The bicarbonate-free solutions were buffered with 9mM Tris OH/Tris Cl (pH 7.4).

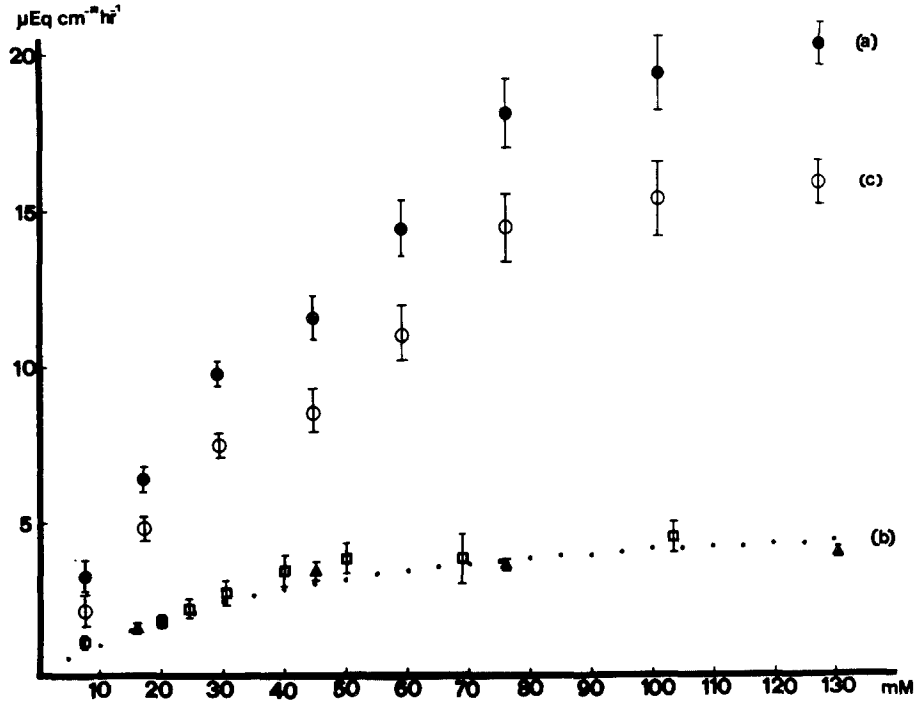


**Fig. 1.** Kinetics of $Cl^-$ influx (45 s) in the presence of bicarbonate in both bathing fluids. The influx is reported as μeq $cm^{-2}$ $h^{-1}$. *a*) Total $Cl^-$ influx from the lumen to the epithelium (8 gallbladders for each point). *b*) $Cl^-$ influx in the absence of $Na^+$ in the lumen (triangles) or in the presence of 25 mM $SCN^-$ (open squares) (5 gallbladders for each point). *c*) $Cl^-$ influx from the lumen to the cell (curve *a* – dotted line *b*). Data are reported as means and standard errors; the dotted line is the theoretical curve interpolating the experimental values of curve *b*. Further details in the text

$cm^{-2}\ h^{-1}$, a value which is significantly lower than 28.0 μeq $cm^{-2}\ h^{-1}$, previously reported (Cremaschi et al., 1979). This has been proved to be due to the absence of any stretching of the epithelium in the present experiments; in fact any stretch was carefully avoided when mounting the preparation as it has been suggested that this increases the paracellular influx (Barry, Diamond & Wright, 1971; Frizzell et al., 1975), which for our purposes is a parasitic influx.

The kinetics obtained is complex. On the other hand, it should include the paracellular influx, although reduced to a minimum for the reason mentioned above. In order to determine this component we measured the influx in the absence of $Na^+$ in the luminal saline for the 45 s measuring time and a preincubation period of 135 s (for this time selected *see below*). Under these conditions $Cl^-$ influx kinetics is reduced to kinetics *b* (open triangles) of Fig. 1. Alternatively, the paracellular influx was measured by replacing 25 mM $Cl^-$ with 25 mM $SCN^-$ which completely inhibits cellular $Cl^-$ influx (Cremaschi et al., 1979). Replacement was effected only during the 45 s measuring time. The kinetics obtained under these conditions is not significantly different from that determined in the absence of $Na^+$ (kinetics *b* of Fig. 1, open squares).

Unexpectedly, the paracellular kinetics is not linear. By the Lineweaver-Burk double-reciprocal plot of all the data obtained in the absence of $Na^+$ or in the presence of $SCN^-$ one obtains a straight line with a correlation coefficient of 0.767 and a highly significant statistical probability. The calculated hyperbola (dotted line in Fig. 1) fits all the experimental points well. Michaelis constant ($K_m$) is 43.3 mM and the maximal influx ($J_{max}$) is 5.8 μeq $cm^{-2}\ h^{-1}$. In order to test whether exchange diffusions or restricted diffusions are present, we bathed the tissue during the 45 min preincubation period with a $Cl^-$-free saline ($Cl^-$ replaced by $SO_4^{2-}$ and mannitol) on both sides and we then exposed the apical side for the 45 s measuring time to a 102.7 mM $Cl^-$ saline containing 25 mM $SCN^-$. Under these conditions we obtained an influx equal to 5.7 μeq $cm^{-2}\ h^{-1}$ (4 gallbladders) which is significantly higher than the control obtained with a normal preincubation ($3.8 \pm 0.6$ μeq $cm^{-2}\ h^{-1}$, 8 gallbladders). The result is in favor of restricted (single file) diffusion of $Cl^-$ in the paracellular pathway which is in agreement with the very low paracellular $Cl^-$ conductance (7% of the total) measured by Barry et al. (1971) in the native unstretched tissue.

Kinetics *c* of Fig. 1 is obtained by subtraction of curve *b* (dotted line) to curve *a*. It is clear that the kinetics remains complex in spite of the elimination of the paracellular component.

### *Time Course of the Effects of $Na^+$ Removal and Bicarbonate Addition*

If, in the presence of bicarbonate in the bathing salines, $Na^+$ was removed on the luminal side for the measuring time (45 s), $Cl^-$ influx decreased significantly from 20.4 to 12.9 μeq $cm^{-2}\ h^{-1}$ (Fig. 2).

A not significantly different result was obtained by reducing the measuring time from 45 to 10 s ($Na^+$ present: $19.8 \pm 0.1$ μeq $cm^{-2}\ h^{-1}$, 4 gallbladders; $Na^+$ absent: $12.9 \pm 0.5$ μeq $cm^{-2}\ h^{-1}$, 4 gallbladders). Thus, a fraction of $Cl^-$ influx appears to be immediatly $Na^+$-dependent.

If $Na^+$ was also removed during a further

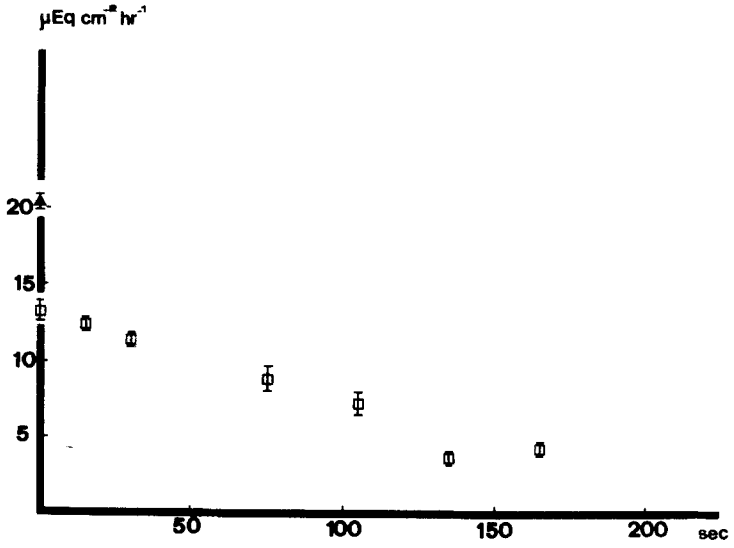


**Fig. 2.** Time course of the total $Cl^-$ influx (μeq $cm^{-2}\ h^{-1}$) after $Na^+$ removal from the lumen (presence of bicarbonate in both bathing fluids). Triangle: control ($Na^+$ present). Open squares: $Na^+$ absent in the lumen during the measuring time (45 s) and for the preincubation time reported in abscissa (seconds). Four gallbladders for each point

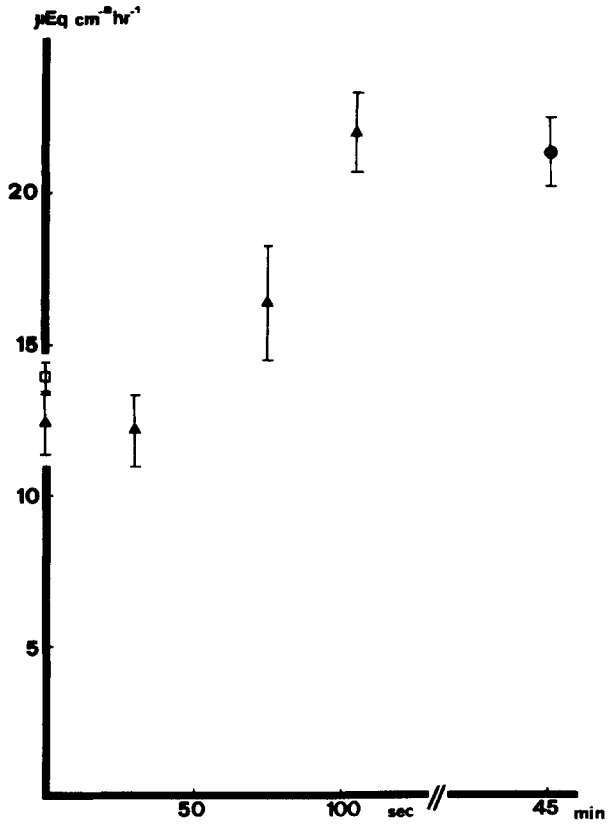


**Fig. 3.** Time course of the total $Cl^-$ influx (μeq $cm^{-2}$ $h^{-1}$) after 25 mM bicarbonate addition to the lumen. Open square: control (bicarbonate absent from both bathing fluids for 45 min and during the measuring time of 45 s). Triangles: bicarbonate present in the lumen during the measuring time (45 s) and for the preincubation time reported in abscissa (seconds). Closed circle: bicarbonate present in both bathing fluids during the measuring time and for 45 min of preincubation time. Six gallbladders for each point

preincubation time, $Cl^-$ influx slowly decreased to reach the minimum of 4.0 μeq $cm^{-2}$ $h^{-1}$ after 135 s of preincubation (Fig. 2). Thus, a second fraction of $Cl^-$ influx appears to be slowly $Na^+$-dependent. The third fraction is $Na^+$-independent and its value is not significantly different from the corresponding value reported in Fig. 1.

If the tissue was preincubated for 45 min in bicarbonate-free saline, $Cl^-$ influx was significantly reduced from the control value of 21.4 to 14.0 μeq $cm^{-2}$ $h^{-1}$ in agreement with what was previously reported (Cremaschi et al., 1979). When 25 mM bicarbonate was introduced on the luminal side, it enhanced $Cl^-$ influx back to the control value only after 150 s (45 s of measuring time + 105 s of preincubation time) (Fig. 3). Thus, the fraction of $Cl^-$ influx dependent on exogenous bicarbonate is only slowly dependent on its presence.

### *Kinetics of $Cl^-$ Influx in the Absence of Exogenous Bicarbonate*

Figure 4 reports the kinetics of $Cl^-$ influx when the tissue was preincubated for 45 min in bicarbonate-free salines (curve *a*). The kinetics remains complex in spite of the absence of the fraction dependent on exogenous bicarbonate. Complexity does not seem to be due to alterations introduced by the particular replacement of $Cl^-$ by $SO_4^{2-}$ and mannitol (open squares) as a not significantly different kinetics was obtained when ethanesulfonate was substituted for $Cl^-$ (closed circles).

When $Na^+$ was absent on the luminal side during the measuring time (45 s), the kinetics de-

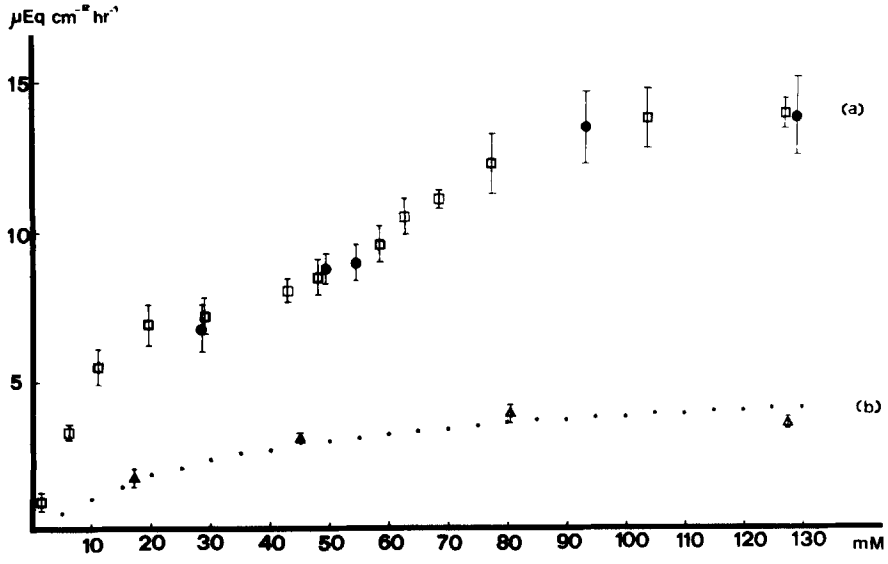


**Fig. 4.** Kinetics of $Cl^-$ influx (μeq $cm^{-2}$ $h^{-1}$) in the absence of bicarbonate in the bathing fluids. *a*) Total $Cl^-$ influx from the lumen to the epithelium; open squares and closed circles indicate that $Cl^-$ was replaced by $SO_4^{2-}$ and mannitol (7 gallbladders for each point) or by ethanesulfonate (4 gallbladders for each point), respectively. *b*) $Cl^-$ influx in the absence of $Na^+$ in the lumen; the dotted line is the theoretical curve interpolating the experimental values (4 gallbladders for each point)

creased to the values of curve *b* (Fig. 4). The outcome did not change if $Na^+$ was absent for a further 135 s. Curve *b* is a hyperbola (dotted line) with $K_m$ 36.3 mM and $J_{max}$ 5.2 μeq $cm^{-2}$ $h^{-1}$ (analyzed by Lineweaver-Burk plot): it is not significantly different from curve *b* of Fig. 1. Thus, in the absence of exogenous bicarbonate, the paracellular component is not affected and the cellular component corresponds to the fraction of $Cl^-$ influx which is immediately $Na^+$-dependent; by contrast, the fraction of $Cl^-$ influx which is dependent on exogenous bicarbonate corresponds to the slowly $Na^+$-dependent fraction.

*Effects of Acetazolamide*

Endogenous cell bicarbonate is likely to be present in the preparation even in the absence of exogenous bicarbonate. So, the tissue was treated on the luminal side with $10^{-4}$ M acetazolamide for the measuring time (45 s). With this concentration the drug specifically inhibits only carbonic anhydrase (Maren, 1977) abolishing the activity of both its cytoplasmic and membrane-bound species (Maren, 1980). Under these conditions $Cl^-$ kinetics was reduced to that reported in Fig. 5 (curve *a*). If one subtracts from it the paracellular component (curve *b* of Fig. 4), a sigmoidal curve is obtained which represents the kinetics of a transport immediately dependent on $Na^+$ and independent of both exogenous and endogenous bicarbonate (Fig. 5, curve *b*). Conversely, if one subtracts from the overall kinetics determined in $HCO_3^-$-free salines (curve *a* of Fig. 4) that determined when acetazolamide is present (Fig. 5, curve *a*), the curve

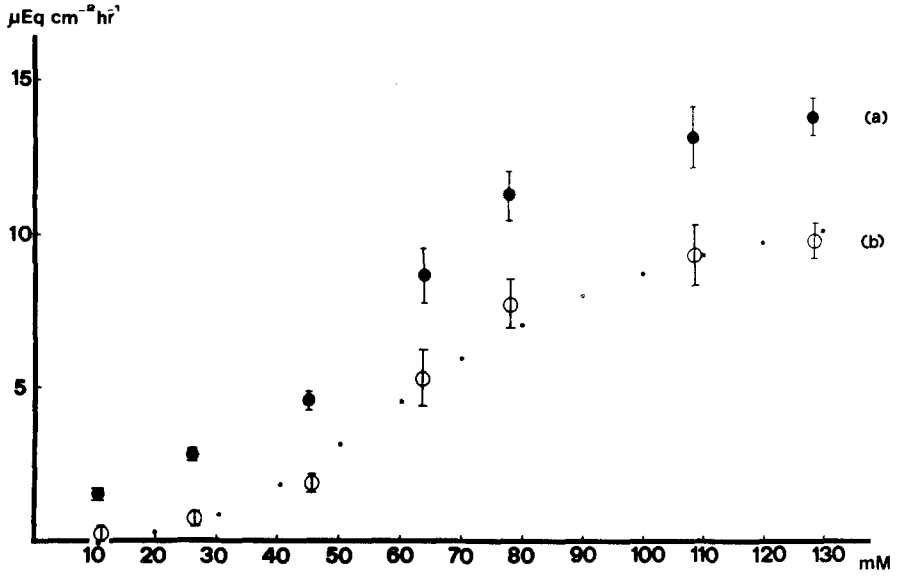


**Fig. 5.** Kinetics of $Cl^-$ influx (μeq $cm^{-2}$ $h^{-1}$) in the absence of bicarbonate in both bathing fluids (45 min + 45 s) and in the presence of $10^{-4}$ M acetazolamide in the lumen (45 s). *a*) Total $Cl^-$ influx from the lumen to the epithelium (4 gallbladders for each point). *b*) $Cl^-$ influx from the lumen to the cell; (curve *a* – dotted line of Fig. 4); the dotted line represents the theoretical curve best interpolating the experimental values

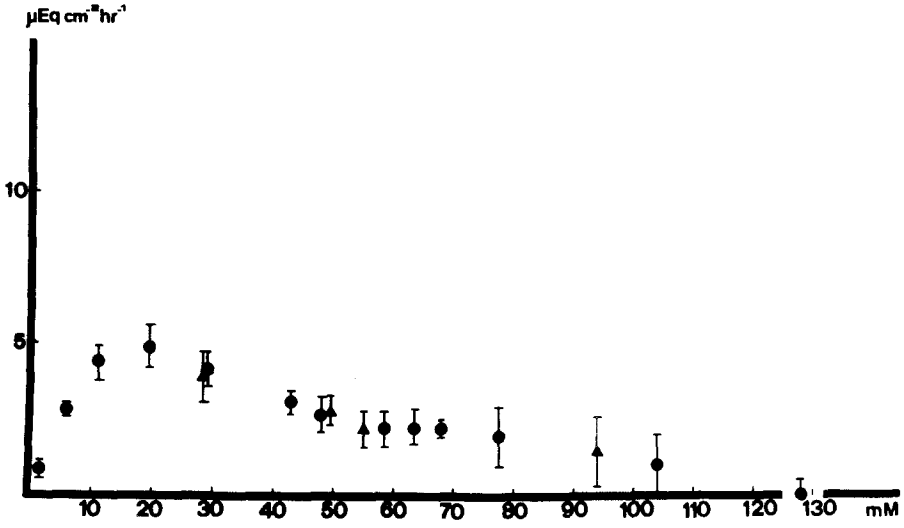


**Fig. 6.** Kinetics of $Cl^-$ influx (μeq $cm^{-2}$ $h^{-1}$) sensitive to $10^{-4}$ M acetazolamide (in the lumen for 45 s) in the absence of bicarbonate in both bathing fluids (curve *a* of Fig. 4 – dotted lines *b* of Figs. 4 and 5). Closed circles and triangles indicate the different replacements of $Cl^-$ ($SO_4^{2-}$ and mannitol or ethanesulfonate, respectively)

of Fig. 6 is obtained; this represents the kinetics of a cellular transport immediately $Na^+$-dependent but also dependent on endogenous bicarbonate.

*Analysis of the Immediately $Na^+$-dependent Transports*

The kinetics of the cellular component immediately $Na^+$-dependent and completely independent of bicarbonate is sigmoidal. The analysis of the sigmoid was performed by the Lineweaver-Burk plot. In this case, as predicted, a straight line was not obtained, but a curve whose intercept with $y$-axis provides $1/J_{max}$ value ($J_{max}=11.6$ μeq $cm^{-2}$ $h^{-1}$). The plot gave a straight line only when $Cl^-$ concentration was raised to an exponent 3 (double reciprocal plot of Hill equation); in this case the correlation coefficient was 0.743 and the statistical probability $<0.01$. The substrate concentration able to halve $J_{max}$ ($S_{0.5}$) was 69.8 mM. In this way the theoretical curve (dotted line in Fig. 5) was also drawn. The analysis was also effected by Hill equation in its logarithmic form:

$$\log \frac{J}{J_{max}-J} = n \log[Cl] - \log K'$$

where $J$ is the influx at $Cl^-$ concentration [Cl], $K'$ is $S_{0.5}^n$ and $n$ is the number of the interacting substrate binding sites. In this case a straight line was obtained with high correlation coefficient ($r=0.928$) and statistical probability ($P<0.01$) (Fig. 7). $S_{0.5}$ turned out to be 69.4 mM and $n$ was 2.95, a value very close to 3.

The kinetics of the cellular component immediately $Na^+$-dependent and also dependent on endogenous cell bicarbonate (Fig. 6) is hyperbolic at low $Cl^-$ concentrations. The analysis was performed both on the first three and four points with equal results ($K_m=8.0$ mM, $J_{max}=6.4$ μeq $cm^{-2}$ $h^{-1}$, $r=0.942$, $P<0.01$). With a luminal $Cl^-$ concentration of about 20 mM a maximal influx is reached (4.8 μeq $cm^{-2}$ $h^{-1}$); then the influx is progressively reduced by increasing further $Cl^-$ concentration and is completely abolished at phy-

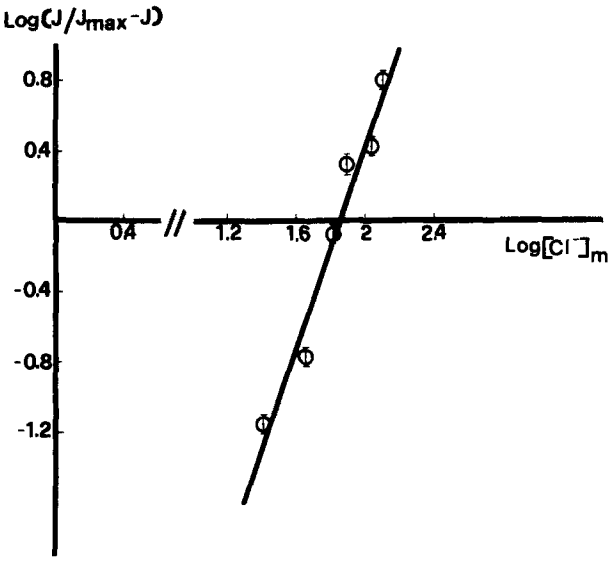


**Fig. 7.** Hill plot of curve *b* in Fig. 5. $J$ is the $Cl^-$ influx (μeq $cm^{-2}$ $h^{-1}$) and $J_{max}$ its maximal value; $[Cl]_m$ is the luminal $Cl^-$ concentration (mM)

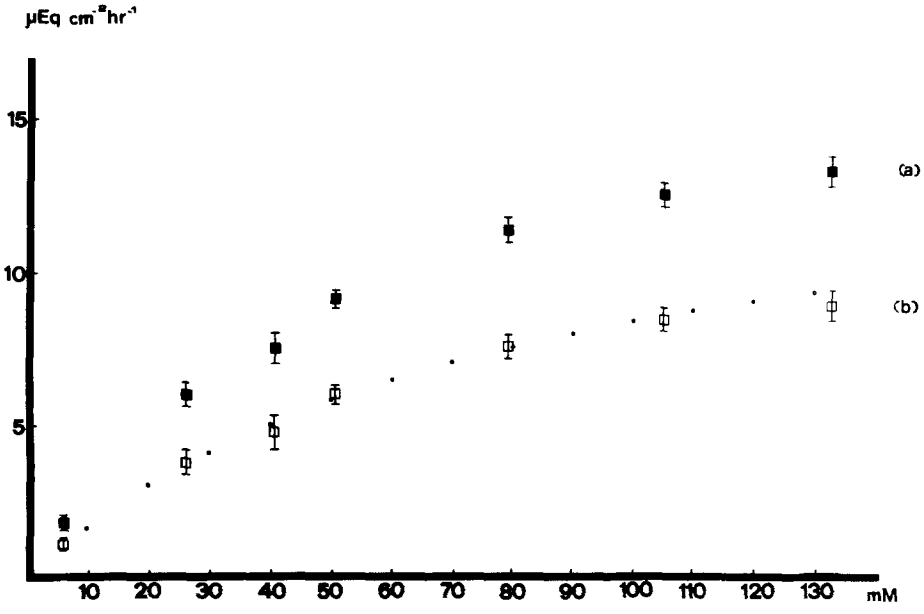


**Fig. 8.** Kinetics of $Cl^-$ influx (μeq $cm^{-2}$ $h^{-1}$) in the presence of bicarbonate in both bathing fluids (45 min + 45 s) and in the absence of $Na^+$ in the lumen during the measuring time (45 s). *a*) Total $Cl^-$ influx from the lumen to the epithelium (4 gallbladders for each point). *b*) $Cl^-$ influx from the lumen to the cell; (curve *a* – dotted line *b* of Fig. 1); the dotted line represents the theoretical curve interpolating the experimental values

siological concentrations (about 130 mM). Thus, the kinetics of this component seems to exhibit a substrate inhibition.

*Analysis of the Slowly $Na^+$-dependent Transport*

The cellular component of $Cl^-$ influx which is slowly dependent on exogenous bicarbonate is also slowly inhibited by the removal of $Na^+$. Thus, the kinetics was obtained by eliminating the immediately $Na^+$-dependent components. $Na^+$ was only removed during the measuring time, in the presence of exogenous bicarbonate. The result is reported in Fig. 8 (curve *a*). When the paracellular component was subtracted, curve *b* was obtained. The analysis with the double-reciprocal plot gave a straight line ($r=0.954$; $P<0.01$) on the basis of which the theoretical hyperbola (dotted line) was drawn. $J_{max}$ turned out to be 15.9 μeq $cm^{-2}$ $h^{-1}$ and $K_m$ 86.4 mM.

## Discussion

The kinetics of the $Cl^-$ influx due to a cotransport with $Na^+$ has been previously examined by Frizzell et al. (1973) and Nellans et al. (1973) for rabbit ileum and by Ahearn and Tornquist (1977) and Ahearn (1978) for the intestine of freshwater prawns. The former group of authors found a hyperbolic kinetics, the latter a sigmoidal curve; on the other hand the cotransport exhibited a stoichiometry of one sodium for each chloride transported in the rabbit ileum, but of two sodium ions for each chloride in the prawn intestine. An electrically neutral cotransport of both ions is a more general finding (Frizzell et al., 1979), but even in this case several possible models, each fitting some of the data obtained, are to be again taken into account, as shown in the Introduction. Thus, the "cotransport of sodium and chloride" could include many different kinds of transfer sharing the property that the two ions are transported necessarily together with a direct or an indirect (but not electrical) coupling.

The results presented here demonstrate that three different kinds of "neutral $Na^+-Cl^-$ cotransport" are present at the apical membrane of the epithelial cells in rabbit gallbladder, since $Cl^-$ influx from the lumen to the cell can be: i) immediately $Na^+$-dependent and completely independent of $HCO_3^-$, ii) immediately $Na^+$-dependent and affected by at least cell endogenous $HCO_3^-$, or iii) slowly $Na^+$-dependent and requiring the presence of exogenous bicarbonate.

Some preliminary observations are important.

First of all, the dependence on $Na^+$ and $HCO_3^-$ is not related to electrical variations as it takes place even under experimental conditions in which no electrical variations are present or, if present, are too small to be able to drive the fraction of $Cl^-$ influx which is affected by the two ions (Frizzell et al., 1975; Hénin & Cremaschi, 1975; Cremaschi et al., 1979; Cremaschi & Meyer, 1982). Under our experimental conditions, in which unilateral changes of $Cl^-$ and $Na^+$ concentrations occur, some electrical variations are involved which in principle could alter the patterns of the kinetics measured; however, simple considerations demonstrate that the relative errors are negligible.[2]

Secondly, the removal of $Na^+$ was certainly complete when it was so required by the experimental condition; this is demonstrated by the fact that the $Na^+$-independent fraction completely overlaps the $SCN^-$-insensitive fraction. Thus, the two fractions of $Cl^-$ influx, the $Na^+$-sensitive and the $Na^+$-insensitive, are correctly discriminated.

Thirdly, when the influx is defined "immediately $Na^+$-dependent," this is based on the fact that: i) an equal influx decrease (measured as μeq $cm^{-2}$ $h^{-1}$) is obtained when $Na^+$ is removed either during a measuring time of 45 or 10 s; ii) in the absence of exogenous bicarbonate the removal of $Na^+$ only during the measuring time (45 s) reduces the influx to the $Na^+$- and $SCN^-$-independent fraction.

Fourthly, the dependence on exogenous or endogenous $HCO_3^-$ may also indicate a sensitivity to the cell pH. Parallel to this dependence a sensitivity to the luminal pH has been shown (Heintze et al., 1981).

Finally, the replacement of $Cl^-$ by $SO_4^{2-}$ is correct as $SO_4^{2-}$ does not enter the cell at the pH of the salines used (Cremaschi & Hénin, 1975).

On this basis, it seems to be reasonable to propose the following model (Fig. 9*a*). The first frac-

[2] Under our experimental conditions the pattern of the kinetics of $Cl^-$ influx could be affected by two different electrical variations: i) that introduced by the reduction in luminal $Cl^-$ concentration (+3.2 mV, serosa positive, as a maximal value, when luminal $Cl^-$ concentration was 5 mM), and ii) that caused by the removal of $Na^+$ in the lumen (−17.5 and −14.3 mV at the luminal $Cl^-$ concentrations of 127.7 and 5 mM, respectively). Since no $Cl^-$ conductance is present in the apical membrane (Hénin & Cremaschi, 1975; Cremaschi & Meyer, 1982), only the paracellular component of the influx may be affected by the PD. However, the paracellular kinetics measured in the absence of $Na^+$ and that determined in the presence of $SCN^-$ are not significantly different, and this is evidence that the influence of the PD introduced by the removal of $Na^+$ is within the limits of experimental errors and that the effect of the much smaller PD caused by the decrease in $Cl^-$ concentration should then be even less.

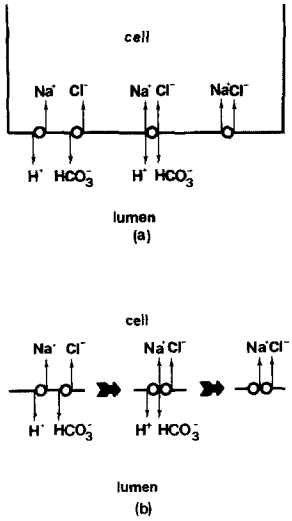


**Fig. 9.** *a*) The three different $Na^+ - Cl^-$ cotransports present in the apical membrane of rabbit gallbladder: a double and indirectly coupled exchange, a double and directly coupled exchange, a true NaCl cotransport. *b*) Hypothetical derivation of the three cotransports from two basal molecular units

tion of the cellular component of $Cl^-$ influx which is immediately $Na^+$-dependent and completely insensitive to bicarbonate, should be due to a true cotransport of $Cl^-$ and $Na^+$ on a single carrier without any exchange with protons and $HCO_3^-$. Its sigmoidal kinetics indicates that three subunits are present in the carrier, cooperating positively with one another. The subunit interaction factors should be strong as is indicated by the integral value of the substrate binding sites found and by the straight line obtained with the Hill plot (weak interactions lead to nonintegral values of $n$ and to a curve in the Hill plot): the consequence is that the carrier should be present in the membrane either in its unloaded form or with all the subunits loaded. Since the cotransport is neutral three $Na^+$ binding sites should correspond to the three $Cl^-$ binding sites. Work is in progress to investigate whether a cross cooperativity also exists among $Cl^-$ and $Na^+$ sites.

The second fraction of the cellular component of $Cl^-$ influx, which is immediately $Na^+$-dependent and sensitive at least to endogenous $HCO_3^-$ (and probably to cell pH), should be due to a cotransport of $Cl^-$ and $Na^+$ on a single carrier with a directly coupled double exchange of $Cl^-/HCO_3^-$ and $Na^+/H^+$. Its complex kinetics shows the presence of a hyperbola from 0 to 20 mM $Cl^-$ and of an inhibition curve due to an excess of substrate concentration from 20 to 130 mM; the inhibition is completed at normal physiological $Cl^-$ concentrations. The hyperbola indicates that only one or even many, but noncooperating, binding sites are present. The substrate inhibition could indicate the presence of more than one binding site, one of them with higher affinity for $Cl^-$. The carrier ($C$) should be able to transfer $Cl^-$ only when this latter site is loaded ($C-Cl$), but should become progressively unable to transport when more than one site is loaded (e.g. $Cl-C-Cl$) (*see* Segel, 1975).

The third fraction of the cellular component of $Cl^-$ influx, which is only slowly $Na^+$-dependent and slowly sensitive to exogenous bicarbonate and luminal pH, should be due to a cotransport of $Cl^-$ and $Na^+$ on two separate carriers, one exchanging $Cl^-/HCO_3^-$, the other $Na^+/H^+$; an indirect coupling of the two antiports should be present mediated by $H^+$, $HCO_3^-$ and carbonic anhydrase. The presence of a $Na^+/H^+$ antiport has been also demonstrated previously (Whitlock & Weeler, 1969; Sullivan & Berndt, 1973; Cremaschi et al., 1979) and the same applies to the $Cl^-/HCO_3^-$ exchange (Heintze et al., 1981). The hyperbolic kinetics of this latter exchange corresponds to that observed for the similar exchange present in the erythrocytes (Gunn, 1979); it indicates that only one or possibly many, but noncooperating, binding sites are present. No substrate inhibition is revealed in the tested concentration range (in the erythrocytes it begins to be present at about 150 mM $Cl^-$ concentration).

It is noteworthy that a selective action of diverse inhibitors on the three transport systems occurs. $10^{-4}$ M acetazolamide is able to inhibit systems 2 and 3, but not system 1. Work in progress shows that $10^{-5}$ M furosemide and $10^{-6}$ M SITS (4-acetamido-4′-iso-thiocyanato-stilbene-2,2′-disulfonic acid) are able to inhibit system 3 (as in erythrocytes), but not systems 1 and 2 (*unpublished results*). Only thiocyanate, among the inhibitors so far tested, can inhibit all three transport systems, competes for the $Cl^-$ binding sites, is very slowly cotransported with $Na^+$, and its transfer is $Na^+$-dependent and stimulated by bicarbonate (Cremaschi et al., 1979).

Among the three transport systems thus characterized only the true cotransport and the double, indirectly coupled, exchange are present at the normal high $Cl^-$ concentrations (about 130 mM); they have low affinities ($S_{0.5} = 69.8$ mM and $K_m = 86.4$ mM, respectively) and large transport capacities ($J_{max} = 11.6$ and 15.9 μeq $cm^{-2}\,h^{-1}$, respectively). Conversely the double, directly coupled, exchange works only at low $Cl^-$ concentrations with a maximum at 20 mM; nevertheless it is not physiologically meaningless. In effect, it must be emphasized that the $Cl^-$ concentration of the bile just

stored in the gallbladder (hepatic bile) is high, but drastically falls with time because of the reabsorption of $Na^+$ salts and water, with $Cl^-$ and $HCO_3^-$ preferentially transported instead of bile acids (cystic bile). Under these conditions a transport with high affinity ($K_m = 8.0$ mM) and able to depend only on cell bicarbonate can support a further reabsorption.

Finally, it is very interesting to emphasize that the three transport systems we have revealed in the apical membrane of rabbit gallbladder surprisingly share properties which gradually lead from system 3 (a double separate exchange) to system 2 (a double exchange on a single carrier) and to system 1 (a cotransport without exchange, on a single carrier). It could be a fascinating hypothesis to suggest that two molecular units, one able to exchange $Na^+/H^+$, the other $Cl^-/HCO_3^-$, are the basis of these three cotransports and that the different properties observed are simply due to the different arrangements undertaken by these two molecules bound together (Fig. 9*b*).

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