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# Carrier-mediated choline uptake by Krebs II ascites cells

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**Krebs II ascites cells have a low affinity uptake system for choline ($K_m$ = 36 μM, $V_m$ = 76 nmol/min per $2 \cdot 10^8$ cells). Choline entered the cells and was rapidly phosphorylated (95% of total intracellular soluble label). Trans acceleration of labeled choline from cells preloaded with radiolabeled choline and postincubated in the presence of unlabeled choline indicates that choline transport in Krebs II ascites cells is carrier mediated. Ethanolamine competed for the choline carrier. The uptake was reduced by hemicholinium-3, iodoacetamide and ouabain. The mechanism of choline transport in Krebs II ascites cells is in agreement with a linear transport model.**

## Introduction

Membrane biogenesis accompanying cellular growth needs an adapted phospholipid biosynthesis [1] and a higher rate of phospholipid turnover has been described in malignant cells [2]. To disturb membrane formation in a rapidly growing tumoral tissue, we have previously studied the action of chlorpromazine [3], a cationic amphiphilic drug and of hemicholinium-3 [4], a potent inhibitor of the choline metabolism, on the phospholipid metabolism of Krebs II ascites cells [5]. This cellular model has been used for several years in our laboratory in studies dealing essentially with phospholipid metabolism [6–9].

As the supply of substrate, for the phosphatidylcholine biosynthesis reactions could be restricted at the point of choline uptake [10] and as a few attempts were made before to study the choline uptake by a tumor cell which does not stem from the nervous system [11], we have focused our study on the choline transport in Krebs II ascites cells.

In eucaryotes, besides the simple diffusion of choline across the plasma membrane, two choline uptake systems acting as saturable mechanisms have been described: a high-affinity sodium-dependent choline transport, highly concentrated in cholinergic neurons [12–18], and a low-affinity choline transport, found in most cells [18–22].

The present communication deals with the characterization of the choline uptake system of Krebs II ascites cells in terms of kinetic features, metabolism of choline, ionic requirement and the effect of metabolic inhibitors or drugs. The experimental test of Krupka and Deves [23] used to distinguish between cyclic and linear transport models provides evidence for a mobile carrier mechanism.

## Materials and Methods

*Labeled and unlabeled compounds.* [*Me*-$^{14}$C]Choline chloride (58 mCi/mmol), [U-$^{14}$C]sucrose (555 mCi/mmol), [2-$^{14}$C]ethan-1-ol-

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

2-amine hydrochloride (44 mCi/mmol) were purchased from the Amersham International, Amersham (U.K.). Radioactivity was determined using a Kontron Intertechnic SL 4000 Liquid scintillation counter.

Choline chloride (99%) was obtained from Aldrich Chemical (F.R.G.); decamethonium bromide and bovine serum albumin, essentially free of free-fatty acid, were from Sigma, St. Louis, MO (U.S.A.); 2-*n*-dibutylaminoethanol and *N*-ethylmaleimide were from Fluka, Buchs (Switzerland). Other reagents of analytical grade were obtained from Prolabo, Paris (France).

*Cell isolation.* Ascitic fluid was harvested from 10–12 female Swiss mice, 9–10 weeks old, which had been intraperitoneally inoculated with ascitic fluid 7 or 8 days before. Cells were supplemented with 2000 units penicillin and 2000 $\mu$g streptomycin per ml before transfer. They were separated from the exudate at $700 \times g$ (10 min) and were washed twice in 3 vol. of a 40 mM Hepes buffer solution (40 mM Hepes, 20 mM $Na_2HPO_4$, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 5 mM KCl, 100 mM NaCl, 11 mM glucose, 0.1 mM albumin, 200 U/ml penicillin, 200 $\mu$g/ml streptomycin, pH 7.34, 300 mosmol/kg). Cells were suspended in choline-free 40 mM Hepes buffer to the appropriate cell density.

*Influx experiments.* Cells ($3.3 \cdot 10^6$ cells/ml) were suspended in 40 mM Hepes buffer containing 0.4 $\mu$Ci [*Me*-$^{14}$C]choline, 400 $\mu$M unlabeled choline, in the presence or in the absence of various effectors. The total volume of the media was 5 ml and the osmolality was adjusted in all experiments at 300 mosmol/kg by varying the NaCl concentration. In experiments where the NaCl concentration was lowered, LiCl was used instead. After incubation at 37°C for various times, cells were separated from the radioactive buffer by centrifugation at $700 \times g$ (10 min) and washed twice in ice-cold Hepes buffer.

*Efflux experiments.* Cells were treated as in influx experiments and they were suspended in fresh 40 mM Hepes buffer to the appropriate density ($3.3 \cdot 10^6$ cells/ml) and incubated at 37°C with or without addition of unlabeled substrate or effector, depending on the experiment. The extracellular medium was isolated as above.

*Extraction and analysis.* Loaded cells were treated with trichloroacetic acid (10% w/v) and the acid-soluble material was placed in a scintillator for total uptake measurement (when the incubation time was not above 15 min). Alternatively, the acid-soluble fraction was extensively washed with ethyl ether and chromatographied on Silicagel 60 precoated plates 0.25 mm thick (Merck) using methanol/0.6% NaCl (w/v)/20% $NH_4OH$ (10 : 10 : 1, by vol.) as solvent [24].

When the extracellular medium was analysed in efflux experiments, the trichloroacetic acid-soluble fraction of the extracellular medium was washed with ether and lyophilised before chromatography according to Vance et al. [24].

Non-specific uptake (diffusion, adsorption, etc....) was measured by adding 50 mM of unlabeled choline to the system in control tubes [4,15]. Isoosmolarity being maintained as already described. Non-specific uptake was always substracted. The same was done for ethanolamine uptake, 50 mM of miscellaneous unlabeled ethanolamine being added in controls.

Lactate dehydrogenase (EC 1.1.1.27) activity was measured according to Wroblewski and La Due [25] using a commercial kit (Boehringer, Mannheim, F.R.G.).

## Results

Previous studies showed that after labeling of the cells with 0.1–1 mM [*Me*-$^{14}$C]choline no significant amounts of radioactivity appeared in phosphatidylcholine until 30 min compared to the total radioactivity entering the cells [26]. Thus, during the first 15 min of exposure to labeled choline, 97% of the total incorporated label was present in acid soluble material. So, we have estimated the rate of choline uptake by the cells by measuring the radioactivity of the acid-soluble pool, based on the assumption that all choline incorporated into phosphocholine and CDPcholine should have passed through the uptake system.

### *Choline uptake by Krebs II ascites cells*

Fig. 1 shows the time-course of accumulation of radiolabeled choline into the acid-soluble material of the cells. Total choline uptake remained linear with time for 30 min, with a rate of $66 \pm 0.8$

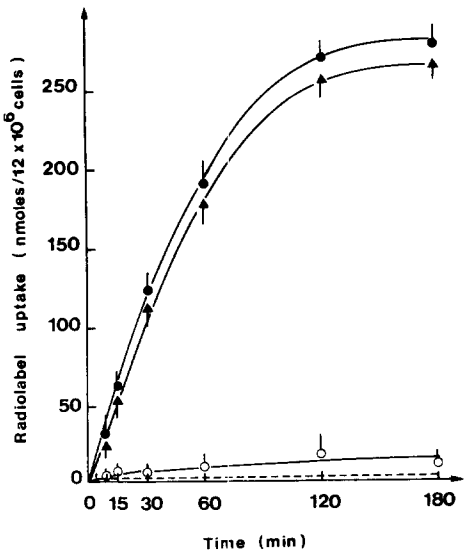


Fig. 1. Accumulation of label by Krebs II ascites cells as a function of incubation time. Krebs II cells ($2.5 \cdot 10^6$ cells/ml) were incubated in 40 mM Hepes buffer in the presence of 0.4 $\mu$Ci [*Me*-$^{14}$C]choline and 1 mM unlabeled choline. At the end of the incubation time, media were treated as described under Extraction and analysis. Total (●), phosphocholine (▲), choline (○) and CDPcholine (----) accumulation values are the means of triplicated determinations.

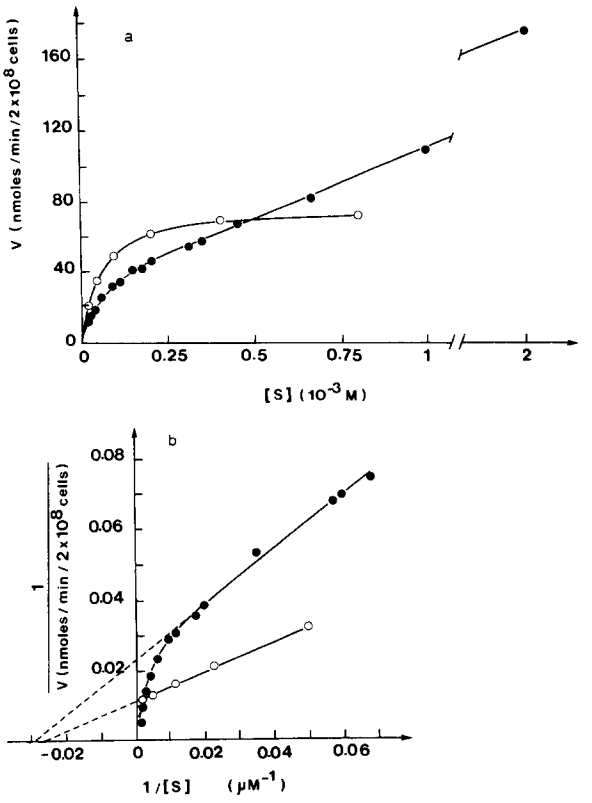


Fig. 2. (a) Plots of the velocities of [*Me*-$^{14}$C]choline and [2-$^{14}$C]ethanolamine accumulation into Krebs II ascites cells, as a function of respective labeled substrate concentrations. (b) Double reciprocal plots. Krebs II cells were incubated 15 min under standard conditions (see Influx experiments) at varying concentrations of unlabeled substrate in the presence of 0.4 $\mu$Ci $^{14}$C-labeled substrate. Velocities of choline uptake (○) and ethanolamine uptake (●) are the means of triplicate determinations. Typical graph taken from four identical experiments.

nmol/min per $2 \cdot 10^8$ cells ($n = 4$). After 30 min the accumulation into the acid-soluble fraction declined in favor of phosphatidylcholine biosynthesis [4]. After 1 h in a medium containing 1 mM choline, the radiolabel accumulated mainly in the form of phosphocholine (95% ± 5% of total acid-soluble label, $n = 4$) with smaller amounts of choline (5%) and less than 0.5% of CDPcholine. By measuring adherent water-space of cell pellets with [U-$^{14}$C]sucrose according to Van Phi and Soling [27], unphosphorylated choline appeared to level off approx. 0.66-times the medium concentration, unlike the phosphocholine which levelled-off at 16.6-times the medium concentration [28].

When the data for specific choline uptake in 15 min were plotted against extracellular choline concentration, the curve indicates that the total choline accumulation approximated Michaelis-Menten kinetics with a $V_m$ of 76 ± 6 nmol/min per $2 \cdot 10^8$ cells and a $K_m$ of 36 ± 6 $\mu$M (Fig. 2a). When the data were plotted according to Lineweaver and Burk, a single straight line was obtained with a correlation coefficient of 0.992 (Fig. 2b).

Under the same conditions, unlike choline accumulation, [2-$^{14}$C]ethanolamine uptake showed a poor tendency to saturate, even at ethanolamine concentration up to 2 mM (Fig. 2a and 2b). Below 0.1 mM, the initial rates of [2-$^{14}$C]ethanolamine incorporation yield a straight line in a Lineweaver-Burk plot, resulting in the same kinetic constant as for choline uptake (34.4 ± 6 $\mu$M).

Nevertheless, the addition of 0.06 mM ethanolamine to the media did result in a decrease of choline accumulation. Fig. 3 clearly shows that inhibition of choline incorporation by ethanolamine was competitive, with a $K_i$ of about 49 $\mu$M.

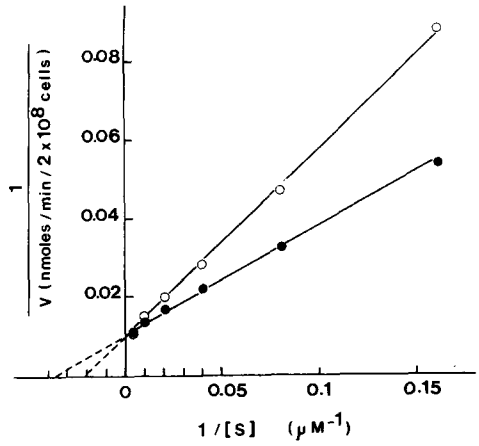


Fig. 3. Effect of 60 μM ethanolamine on [*Me*-$^{14}$C]choline accumulation. Cells were incubated under standard conditions (see Influx experiments) for 15 min with (○) or without (●) 60 μM unlabeled ethanolamine in the extracellular media containing 6.25 to 200 μM [*Me*-$^{14}$C]choline (0.4 μCi). Each point is the mean of triplicated determinations.

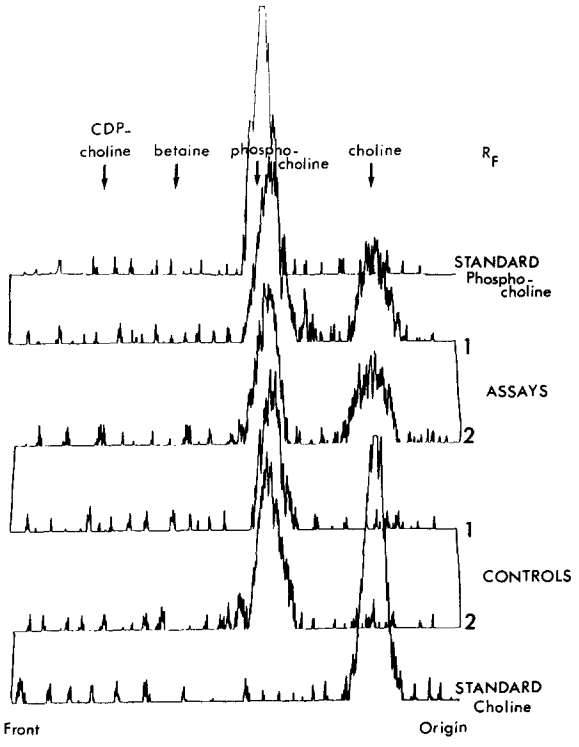


Fig. 4. Scanning radiochromatogram of the products of counter-transport. Cells were preloaded ($3.3 \cdot 10^6$ cells/ml) by incubation for 1 h in 40 mM Hepes buffer containing 800 μM radiolabeled choline (0.2 μCi/ml). Following preloading, cells, in duplicate, were washed twice with cold choline-free Hepes buffer and transferred to fresh choline-free buffer (Controls 1 and 2) or to Hepes buffer supplemented with 5 mM unlabeled choline (Assays 1 and 2). After 0, 20, 40, 60, 90 min, the supernatants 700 g (10 min) were treated as under Materials and Methods (Extraction and analysis of the extracellular medium). After chromatography, the radioactivity of the plots was estimated by radiochromatography scanning.

This is in the same range of magnitude as the $K_m$ previously determined for choline uptake (see above).

*Trans-acceleration of choline transport*

Fig. 4 shows the radioactivity scan of the thin-layer chromatography of the products present in the extracellular media after incubation of [$^{14}$C]choline preloaded cells in the presence of 5 mM choline (assay), or in the absence of choline (control). Only two radioactive peaks, comigrating with choline and phosphocholine standards, respectively, could be identified. In both experiments, phosphocholine radioactivity was the same in controls and assays (3% of total label present in preloaded cells), and certainly corresponded to cell lysis, time-identical values were measured for lactate dehydrogenase release. The only difference between assay and control lanes was found for choline radioactivity, which was increased in assays.

Fig. 5 reports the time-course of [$^{14}$C]choline release from preloaded cells upon addition of 5 mM choline. It is clear that choline addition promoted a 5-fold faster release of [$^{14}$C]choline compared to controls.

*Effects of ions, metabolic inhibitors and drugs*

Early in this work, we have studied the effects of ions on choline uptake by Krebs II cells, in order to optimise the Hepes buffer composition, and investigate the ionic requirements. Fig. 6 shows that there is no absolute requirement of $Ca^{2+}$, $Mg^{2+}$, $K^+$ and $Na^+$, since in extracellular media free of one of these ions, the rate of uptake was never lower than 28 nmol/min per $2 \cdot 10^8$ cells. When $Na^+$ was replaced by $Li^+$ [19,29,30] the effects of exogenous sodium were very low and constant between 0 to 50 mM, while a 20% in-

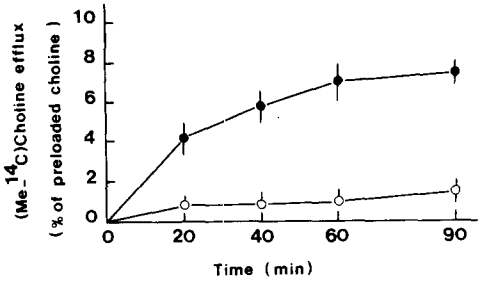


Fig. 5. Efflux of [*Me*-$^{14}$C]choline by counter-transport. [$^{14}$C]Choline is determined after the step of Fig. 4. Each point of the figure represents the % of the radioactivity of choline released by the counter-transport alone. The calculation is based on: dpm of the choline spot: $C$; dpm of the phosphocholine spot: $P$; dpm of intracellular choline at zero time: $T$; dpm of extracellular choline due to cellular lysis: $C \times P/T$. Values on ordinate: $100 \times C(1 - P/T)$. Choline released ± S.E. ($n = 3$) is determined upon addition of choline-free Hepes buffer (○) and upon addition of 5 mM unlabeled choline (●).

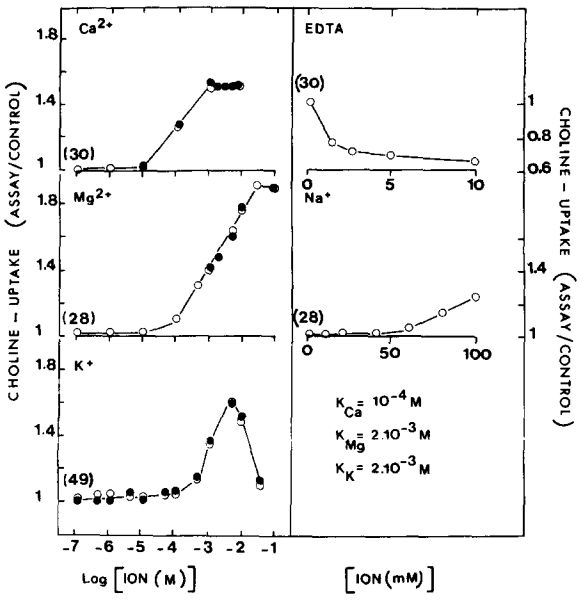


Fig. 6. Effects of ions on choline uptake. Cells were incubated under standard conditions of Influx experiments for 15 min. After incubation, cells were treated as described under Extraction and analysis. Curves are drawn with results of two experiments (○ and ●) for $Ca^{2+}$, $Mg^{2+}$, $K^+$ and with the mean of three determinations for $Na^+$. $K$ is the half maximal activation constant. Values in parenthesis correspond to rate (expressed in nmol/min per $2 \cdot 10^8$ cells) of choline uptake in the medium without the corresponding ion.

crease was observed with 100 mM. However, choline uptake displayed a partial dependence on $K^+$, $Ca^{2+}$ and $Mg^{2+}$, because when these ions were alternatively replaced by sodium, the accumulation of choline was reduced by about 34, 38 and 48%, respectively. This is particularly critical for the potassium concentration which is an activator of choline uptake between $10^{-2}$ and $10^{-3}$ M. The extracellular concentrations, corresponding to maximal activation of choline uptake, were chosen for the Hepes buffer composition.

Table I shows the effects of some metabolic inhibitors and drugs. Ouabain (0.1 mM), an inhibitor of adenosine triphosphate (ATP) phosphohydrolase ($(Na^+ - K^+)$-ATPase, EC 3.6.13), lowered the uptake by about 32%, whereas 2,4-dinitrophenol (0.1 mM), a well-known uncoupler of oxidative phosphorylation was ineffective.

Incubation with iodoacetamide (1 mM), which inhibits glycolysis and oxygen consumption and decreases ATP, significantly lowered the total choline accumulation by 77%.

Lowering the temperature affected the accumulation of choline. Incubation at 4°C reduced the choline uptake by 90%. When the incubation temperature was lowered from 37°C to 27°C, the rate of [*Me*-$^{14}$C]choline uptake decreases with a $Q_{10}$ value of $2.9 \pm 0.1$ ($n = 5$).

The structural analogues, dibutylaminoethanol (1 mM), decamethonium (1 mM) and hemicholinium (HC-3: 0.2 mM) [26], reduced the specific uptake of choline by 14, 50 and 32%, respectively.

*N*-Ethylmaleimide (1 mM) inactivated [$^{14}$C]-choline uptake in Krebs II cells about 88% in 1 min, this displaying a 5-fold higher potency compared to erythrocytes [31].

### *Class of transport mechanism of choline by Krebs II cells*

Two essential mechanisms for active transport have been proposed. In the first one, substrate sites in the free carrier are simultaneously exposed on the two membrane surfaces (linear mechanism), while in the other one, a substrate site is alternatively exposed on opposite sides (cyclic mechanism).

We have used the kinetic test described by Krupka and Deves [23] in order to discriminate between these two classes of transport mecha-

TABLE I

EFFECTS OF METABOLIC INHIBITORS AND DRUGS ON CHOLINE ACCUMULATION INTO KREBS II ASCITES CELLS

The accumulation of [*Me*-$^{14}$C]choline was examined as described under Influx experiments, after preincubation for 10 min at 37°C in the presence of various metabolic inhibitors or drugs: after preincubation for 2 h at 37°C in the absence of glucose; after preincubation for 1 min at 37°C in the presence of *N*-ethylmaleimide. The reaction was stopped with 10 mM β-mercaptoethanol. Values are the means of four determinations. n.s., no significant.

| | Concn. (μM) | [*Me*-$^{14}$C]Choline accumulation (percent of control) |
|---|---|---|
| Glucose omission | | 90 n.s. |
| Potassium ferricyanide | 1000 | 90 n.s. |
| 2,4-Dinitrophenol | 100 | 90 n.s. |
| Iodoacetamide | 1000 | 23 ** |
| Ouabain | 100 | 68 ** |
| Hemicholinium-3 | 200 | 68 ** |
| Ethanolamine | 50 | 95 * |
| Dibutylaminoethanol | 1000 | 86 ** |
| Decamethonium | 1000 | 50 ** |
| *N*-Ethylmaleimide (1 min) | 1000 | 12 ** |
| *N*-Ethylmaleimide (1 min) | 100 | 38 ** |

* $p < 0.05$ ($n = 4$).
** $p < 0.001$.

nisms. This test can account for active and facilitated transport, as well as for accelerated exchange, counter-transport and hyperbolic substrate saturation curves.

In our study, two non-transported substrate analogs have been used:

(1) a non-penetrating quaternary ammonium ion, decamethonium, which would only bind on the outer surface of the membrane [31];

(2) a lipid soluble tertiary amine, 2-di-*n*-butylaminoethanol, which binds only on the inner surface of the membrane [23].

The relationship between the inhibitions produced by each inhibitor alone and that with both inhibitors is the following (Krupka and Deves):
Cyclic mechanism: (Eqn. 1)

$$\left[\frac{V}{V_{IoLi}}-1\right]=\left[\frac{V}{V_{Io}}-1\right]+\left[\frac{V}{V_{Li}}-1\right] \quad (1)$$

Linear mechanism: (Eqn. 2)

$$\left[\frac{V}{V_{IoLi}}-1\right]=\left[\frac{V}{V_{Io}}-1\right]+\left[\frac{V}{V_{Li}}-1\right]+\left[\frac{V}{V_{Io}}-1\right]\left[\frac{V}{V_{Li}}-1\right] \quad (2)$$

where $V$ is the transport rate in the absence of any inhibitor, $V_{Io}$ and $V_{Li}$ are the transport rates in the presence of inhibitors (decamethonium and 2-*n*-dibutylaminoethanol, respectively) and $V_{IoLi}$ is the transport rate when both inhibitors are present.

The observed rate (Table II) with both inhibitors present ($V_{IoLi}$) was found to coïncide with the predicted value of the one-site exposure mechanism, where the inhibition is a function of the sum of inhibitor concentrations. The observed rate is very different from the predicted value of the two-site exposure mechanism, where the inhibition is a function of the product of inhibitor concentra-

TABLE II

TEST OF KRUPKA AND DEVES

Rates of choline uptake were determined in the presence of 0.4 μCi [*Me*-$^{14}$C]choline and 20 μM unlabeled choline, under standard conditions (see Influx experiments.) In the absence of inhibitor ($V$), in the presence of 1.5 mM decamethonium ($V_{Io}$), in the presence of 7.5 mM di-*n*-butylaminoethanol ($V_{Li}$), in the presence of both inhibitors at the same concentrations ($V_{IoLi}$). These concentrations are the inhibitor concentrations required to reduce the rate of choline entry by half at a given substrate concentration [28]. The predicted values of the rate in the presence of both inhibitors at once, $V_{IoLi}$, are calculated from the rate in the presence of each inhibitor alone $V_{Io}$ and $V_{Li}$, on the basis of Eqn. 1 or Eqn. 2. Standard deviations were calculated on four different experiments.

| Rates of choline entry | | | | | |
|---|---|---|---|---|---|
| Experimental values | | | | Predicted values of $V_{IoLi}$ | |
| $V$ | $V_{Io}$ | $V_{Li}$ | $V_{IoLi}$ | One-site | Two-site |
| 25.61 ± 2.21 | 10.47 ± 0.36 | 16.84 ± 0.90 | 8.41 ± 0.12 | 8.62 ± 0.24 | 6.88 ± 0.02 |

tions [23]. In the latter case the difference between the experimental and predicted values is greater than the standard deviation of the difference by a factor 12.7 ($p < 0.001$).

## Discussion

Choline enters the Krebs II cells via a carrier mediated mechanism since it fulfills a number of well-established criteria [32]. The uptake is saturable ($K_m = 36$ μM, $V_m = 76$ nmol/min per $2 \cdot 10^8$ cells), has a characteristic temperature coefficient ($Q_{10} = 2.9$), is inhibited by structural analogues (HC-3, ethanolamine, dibutylaminoethanol, decamethonium) and by chemical reagents also active as enzyme poison (*N*-ethylmaleimide). Furthermore, it displays the phenomenon of counter-transport. Because of its relatively high affinity constant and its sodium-independence, the choline carrier of our cellular model is a low affinity system [12], like in cultured rabbit lenses [20], rat diaphragm [21] and guinea pig lung [22].

Energy dependence of the system is difficult to establish despite its thermodependence ($Q_{10} = 2.9$), because the inhibitions observed with ouabain or with iodoacetamide could be subsequent to the inhibition of a $Na^+$,$K^+$-dependent ATPase. On the other hand, extracellular $Na^+$ can be completely substituted by $Li^+$, and $K^+$ omitted, without suppressing choline uptake, which is not consistent with the upper hypothesis. The energy required for choline uptake should not be provided by glycolysis, because dinitrophenol has no significant effect, which corroborates the results obtained in glucose-free experiments. Moreover, an active carrier system can act in the absence of metabolic energy like a facilitated diffusion transport system.

The choline taken up was rapidly concentrated through choline kinase [4] as phosphocholine (95% of total acid soluble label and about 16-fold the extracellular concentration [28]). Compared to the phosphocholine pool sizes of other vertebrate cells (0.05 to 2 mM) [10], the Krebs II ascites cells are more able to take up extracellular choline by early phosphorylation.

Unlike choline, phosphocholine cannot readily escape the cell. In the efflux trans-infini situation of Fig. 5, free choline alone was released by the carrier system. This counter-transport of choline is supplied by free choline not yet metabolised, or choline coming from phospholipid metabolism [10,33]. This efflux is not increased with increasing concentration of 'accelerative' unlabeled choline over 5 mM [28]. The trans-acceleration phenomenon is generally considered as the best single criterion for a carrier facilitated transport mechanism [10,32,35] and it avoids possible ambiguities resulting from the phosphorylation. In our experimental model, it cannot excess a few percentage of the intracellular label, i.e. the pool size of free choline.

Fig. 3 suggests that the choline carrier might be partially involved in the uptake of ethanolamine. The kinetics of ethanolamine uptake (Figs. 2a, 2b) revealed a saturability of the transport only at low extracellular concentrations, with a $K_m$ essentially identical to that for choline uptake. From other related studies [16,20,26,36,37], it is not clear whether choline and ethanolamine are transported by the same or by different mechanisms. Krebs II cells choline transport sites might bind ethanolamine analogs and choline analogs [28], but we have no evidence for the co-transport of ethanolamine by the choline carrier.

In a previous work on the mechanism of glucose and choline transport in erythrocytes, Krupka and Deves [23] presented a kinetic test. This test allowed us to define the mechanism of choline transport by Krebs II cells as a cyclic one, which means that the substrate site of the carrier oscillates between the two membrane surfaces. It disappears from one of the two membrane faces, before appearing on the other side. It is distinguished from the linear mechanism, in which free sites of the carrier are simultaneously accessible on both membrane surfaces [23]. So Krebs ascites cells, although very different from the erythrocytes, possess the same cyclic transport mechanism for choline, which enhances the hypothesis of the ubiquity of this transport mechanism of choline.

Krebs II ascites cells have a considerable capacity to transport choline, even at 10 μM and to concentrate it in a large pool of phosphocholine. The choline carrier would not be a rate-limiting factor in phospholipid synthesis in these tumoral cells.

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