

Concentrative Accumulation of Choline by Human Erythrocytes

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ABSTRACT Influx and efflux of choline in human erythrocytes were studied using ^{14}C -choline. When incubated at 37°C with physiological concentrations of choline erythrocytes concentrate choline; the steady-state ratio is 2.08 ± 0.23 when the external choline is $2.5 \mu\text{M}$ and falls to 0.94 ± 0.13 as the external concentration is raised to $50 \mu\text{M}$. During the steady state the influx of choline is consistent with a carrier system with an apparent Michaelis constant of $30 \times 10^{-6} \text{ M}$ and a maximum flux of $1.1 \mu\text{moles per liter cells per min}$. For the influx into cells preequilibrated with a choline-free buffer the apparent Michaelis constant is about $6.5 \times 10^{-6} \text{ M}$ and the maximum flux is $0.22 \mu\text{mole per liter cells per min}$. At intracellular concentrations below $50 \mu\text{mole per liter cells}$ the efflux in the steady state approximates first order kinetics; however, it is not flux through a leak because it is inhibited by hemicholinium. Influx and efflux show a pronounced exchange flux phenomenon. The ability to concentrate choline is lost when external sodium is replaced by lithium or potassium. However, the uphill movement of choline is probably not coupled directly to the Na^+ electrochemical gradient.

INTRODUCTION

Choline is a precursor not only for acetylcholine but also for phospholipids that are found in intracellular membranes and in the cell membrane. It is therefore not surprising to find in various tissues specific transport systems facilitating the entry of choline into cells. Choline transport systems exist in ganglia (1), in the squid axon (2), and in erythrocytes (3). Studying the uptake of choline by brain slices Schuberth, Sundwall, Sörbo, and Lindell (4) found that the concentration of choline in the tissue can increase above that in the incubation medium but it has not been investigated to what extent the transmembrane potential contributes to this concentration ratio; however, the effects of anoxia and metabolic inhibitors suggest that this transport system requires energy. Removing the external sodium reduces the ability of brain slices to concentrate choline (5). There is also evidence for active transport of choline in the kidney (6, 7) and the choroid plexus can concentrate various quaternary ammonium ions (8).

The experiments presented here establish that erythrocytes can concentrate choline against an electrochemical gradient. The affinity of the carrier for choline is well within the physiological concentrations of choline (9) but there is no obvious physiological role for this transport system: erythrocytes can incorporate ^{32}P -orthophosphate into some phosphatides (10) but there is no evidence for a *de novo* synthesis of phosphatidylcholine *in vitro* (3, 10, 11). The choline transport system in erythrocytes may therefore be vestigial and more effective in earlier stages of erythropoiesis. It is also similar to the choline transport found in the nervous tissue. The present experiments were carried out because in erythrocytes the parameters of this transport system can be studied rather thoroughly: erythrocytes survive well and are a reasonably homogeneous preparation.

A brief account of part of this work has already appeared (12).

METHODS

Bank blood and fresh blood were used. For each experiment the source of the blood will be indicated in the text. Fresh blood (40 ml) was withdrawn into a syringe containing about 500 IU heparin. Bank blood was drawn into a citrate dextrose mixture (giving 0.5 g acid citrate per 100 ml of blood and adding 0.6 g of glucose to 100 ml of blood), stored at 5°C, and used within 3 days after withdrawal from the donor.

The blood was centrifuged for 10 min at about 1400 *g*, plasma and buffy coat were removed, and the red cells washed four times in 3 volumes of a solution containing NaCl 147 mM, KCl 4 mM, MgCl₂ 1 mM, CaCl₂ 2.2 mM. Tris HCl-Tris base (Tris (hydroxymethyl) aminomethane) 5 mM to give pH 7.4 at 37°C, 0.1% glucose, and 0.02% chloramphenicol. After each wash and centrifugation the topmost layer of cells was removed with the supernatant, so that the four washes reduced the red cell volume by about 30%. Whenever a measured volume of "packed" cells was to be transferred to an incubation medium the cells were centrifuged for 20 min at 1400 *g*.

Influx Experiments 50 ml incubation medium in a 200 ml Erlenmeyer flask were shaken in a water bath at 37°C. The incubation medium had the same composition as the wash solution except that labeled and unlabeled choline were added to it. ^{14}C -choline (35 mc/mM) was present at concentrations giving between 40,000 and 60,000 dpm per ml. Only when the total concentration of choline chloride was 1 mM or more was an equivalent amount of NaCl omitted. At a known time 2 ml of packed cells were added to a flask and the pipette was rinsed several times with the incubation medium. Usually six flasks were incubated simultaneously and the cells added at 1 min intervals. 5 ml aliquots were removed at various times in the same order as that in which blood was added and again at 1 min intervals and were placed in centrifuge tubes cooled in ice water. The samples were then centrifuged at 1400 *g* for 5 min, the supernatant withdrawn, and the cells washed three times with 5 ml ice-cold buffer containing 1 mM unlabeled choline. After the last supernatant was withdrawn the cells were precipitated with 2 ml 5% trichloroacetic acid containing 1 mM choline chloride. (Preliminary experiments had shown that the recovery of added ^{14}C -choline from the supernatant of a TCA precipitate prepared in this way

is between 92 and 104 %.) After centrifugation 1 ml of the supernatant was transferred to a counting vial. Preliminary experiments in which the supernatant from the last washing was counted showed that the fluid trapped between the cells after the last washing contained none or negligible amounts of radioactivity. At the beginning and at the end of the experiment duplicate aliquots of the cell-free incubation medium were removed for counting.

Two types of influx experiments were performed: influx during the steady state and influx into "choline-free" cells.

To measure influx during the steady state, washed cells were equilibrated overnight at 37°C with various concentrations of unlabeled choline. Next morning the cells were washed and incubated in solutions which had the same composition as the equilibration media except that some of the unlabeled choline was now replaced by ^{14}C -choline. The assumption that during the overnight incubation the cells had reached the steady state with respect to choline is based on preliminary experiments in which red cells were equilibrated in the same way but with labeled rather than with unlabeled choline. The intracellular radioactivity showed no change with time when measured at 12, 16, and 20 hr provided the choline concentration in the medium did not exceed 50 μM . For these long incubation periods the blood was in slowly rotating (about 1 rpm) polythene bottles and the hemolysis was less than 3%.

The second type of influx experiments measured the flux of choline into choline-free cells. For these experiments cells were equilibrated overnight at 37°C with a choline-free buffer; the hematocrit was 5% or less. The influx of labeled choline was measured as described above.

With influx experiments errors could arise through loss of radioactivity from the cells during the washing, even though this was carried out with ice-cold solutions and the cells were centrifuged in the cold. The washing took 30–40 min and it is estimated that, despite the high Q_{10} (see Table III), cells may lose up to 5% of their radioactivity during that time.

Hemolysis was estimated by measuring the optical density of the supernatant at 539 $\text{m}\mu$ and comparing it with suitable standards prepared from lysed cells. When the hemolysis exceeded 5% the experiment was discarded, otherwise a correction was applied.

Efflux Experiments Cells were loaded by incubating them for 16 hr at 37°C (hematocrit 10%) in solutions containing various concentrations of choline. As with the influx experiments the concentration of ^{14}C -choline was always constant; the total concentration of choline was varied by adding appropriate amounts of unlabeled choline. The cells were then washed three times with 5 volumes of ice-cold nonradioactive solution and centrifuged in the cold. Immediately after the third wash 0.2 ml of the packed cells was added at 1 min intervals to 20 ml efflux medium and was then shaken in a water bath at 37°C. At various times—and again at 1 min intervals—2 ml aliquots were removed into ice-cold centrifuge tubes and centrifuged for 5 min at 1400 g in the cold; 1 ml of the supernatant was then transferred to a counting vial. At the beginning and at the end of the experiment aliquots of the mixture, i.e. cells plus external medium, were removed, the cells precipitated by adding an equal volume of 10% TCA, and after centrifugation 1 ml of the supernatant placed into a counting vial.

When efflux was to be measured in the steady state, the total concentrations of choline in the loading medium and in the efflux medium (containing only unlabeled choline) were the same.

In some experiments the radioactivity of the cells was determined as well as that of the external medium and it was found that the rates of efflux calculated from the two measurements agreed within 5%. As with the influx experiments, the degree of hemolysis was determined, and, when necessary, the appropriate correction made.

Calculation of Fluxes In the steady state, i.e. when the intracellular and extracellular concentrations of choline remain constant, the flux of choline is the product of the rate constant describing the exchange of radioactivity and the concentration of choline in the labeled compartment. Also, assuming a homogeneous population and a two compartment system, the exchange of radioactivity between intra- and extracellular compartments should follow exponential kinetics. For efflux experiments (hematocrit 2-3% so that the backflux of label can be neglected) this means that a plot of logarithm $\frac{C_t}{C_{t=0}}$ against time should give a straight line with the slope of k , where C_t is the radioactivity in the cells at various times, $C_{t=0}$ the radioactivity in the cells at the beginning of the incubation, and k the rate constant. However, plotting results in this way—samples were usually taken at 10, 30, 60, 120, and 180 min—revealed that the flux of ^{14}C -choline at any choline concentration cannot be described by a single rate constant. The curves were analyzed as suggested by Creese, Neil, and Stephenson (13) and it became obvious that the deviations from exponential kinetics are those associated with a nonhomogeneous population where the logarithms of the rate constants show a normal distribution. In such a situation one can either determine the mean rate constant or settle on an initial rate constant. In this paper the initial rate constant was calculated from the efflux of radioactivity during the first 10 min using the equation $k = 0.1 \ln \frac{C_{t=0}}{C_{t=10}} \text{ min}^{-1}$. For some experiments mean rate constants and initial rate constants were calculated; they are compared in the text.

The mean rate constant for the influx is obtained from a graph of logarithm $1 - \frac{C_t}{C_{\text{eq}}}$ against time when C_{eq} is the intracellular radioactivity at equilibrium. The initial rate constant for the influx can be calculated from the equation

$$k = 0.1 \ln \frac{C_{\text{eq}}}{C_{\text{eq}} - C_{t=10}} \text{ min}^{-1}.$$

The fluxes during the steady state were calculated from the initial rate constant because in situations in which the cells are *not* in the steady state, e.g. experiments measuring the efflux from cells with the same intracellular concentration of choline into media containing different concentrations of choline, only the initial rates can be calculated using the same equations without introducing much error. The errors that might arise from this failure to correct for changes in specific activity during the first 10 min are discussed in the text together with the relevant experiments.

Determination of the Hematocrit To express fluxes and intracellular concentra-

tions in terms of "per volume of cells" the hematocrit was determined for each suspension in duplicate: after 5 ml aliquots were centrifuged for 10 min at 1400 *g*, 4 ml of the supernatant was removed: the concentrated suspension was then mixed and its hematocrit determined with the MSE Minor microhematocrit accessory rotor, (Measuring & Scientific Equipment Ltd., London) centrifuging for 20 min at 6000 *g*.

Counting 10 ml of Bray's scintillator (14) were added to each vial. Each sample was counted twice for 10 min in a Nuclear Chicago liquid scintillation counter. The quenching was estimated from the channel ratio using a standard curve and the counts were converted to disintegrations per min (dpm).

Materials 35 mc/mm choline chloride (methyl ^{14}C) was obtained from the Radiochemical Centre, Amersham, England. The isotope was eluted from the paper with water and the solution stored at -5°C . Each batch was usually used up within 4 wk; if it was used beyond that time the purity of the ^{14}C -choline was examined by chromatography. The recovery of radioactivity as choline was usually between 98 and 103% and never less than 96%.

Unlabeled choline chloride was obtained from Hopkin & Williams, Ltd., (Essex, United Kingdom), and recrystallized from absolute alcohol.

Hemicholinium (HC-3) was a gift from Professor F. C. MacIntosh, McGill University.

Tris base and Tris HCl were obtained from Sigma Chemical Company. All other chemicals were analar grade from Hopkin & Williams, Ltd.

RESULTS

The Influx of Choline The relation between influx of choline into erythrocytes and external concentration of choline was studied in two ways. In the first series of experiments the cells were incubated overnight in a choline-free buffer, in the second series the cells were preequilibrated with various concentrations of unlabeled choline so that the influx of ^{14}C -labeled choline measured the flux in the steady state. In Fig. 1 results from two such experiments are shown and compared with Michaelis-Menten kinetics. The apparent Michaelis constant and the saturating flux are both increased by a factor of about five when the cells are preloaded with choline. The influxes were calculated from the influx of labeled choline during the initial 10 min. During this time the backflux of ^{14}C -choline is so small that dilution of the label by the intracellular unlabeled choline cannot account for the differences in influx rates. The values for K_m and V_m for the upper curve in Fig. 1 are obviously doubtful because experimental points have been obtained only for choline concentrations up to 50 μM . The influx in the steady state at higher choline concentrations was not measured because experimental data to be discussed later suggest that cells preincubated at choline concentrations above 50 μM may not reach the steady state in 16 hr.

Distribution of Choline in the Steady State When the influx of ^{14}C -choline was followed over several hours until the intracellular radioactivity did not

change any more with time, it became clear that the distribution of radioactivity in the steady state is dependent on the concentration of choline. These experiments, however, involve long periods of incubation and are therefore complicated by hemolysis, especially when bank blood is used. The distribution of radioactivity in the steady state can be measured after shorter over-all incubation if cells are not preequilibrated with unlabeled choline but are immediately incubated in solutions containing label and various concentrations of choline. To determine the distribution of choline in the steady state, cells were therefore incubated overnight and intracellular radioactivity determined after 16 and 20 hr. Using fresh cells in these experiments the hemolysis

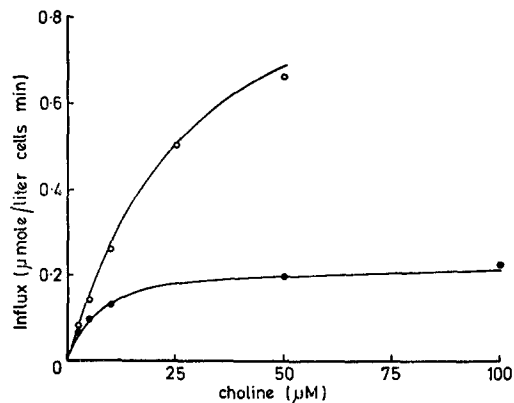


FIGURE 1. Influx of choline during the steady state (open circles) and into cells pre-equilibrated with a choline-free buffer (filled circles). Both lines are theoretical curves for Michaelis-Menten kinetics. The upper one was calculated with $K_m = 30 \mu\text{M}$ and $V_m = 1.1 \mu\text{moles per liter cells per min}$, the lower one with $K_m = 6.5 \mu\text{M}$ and $V_m = 0.22 \mu\text{mole per liter cells per min}$. Bank blood from one donor was used.

was never more than 3% and usually less than 2%. Provided the concentration of choline in the incubation medium did not exceed $50 \mu\text{M}$ the, values for intracellular radioactivity at 16 and 20 hr did not differ significantly and it was assumed that the cells had reached a steady state. Attainment of a steady state with respect to radioactive choline implies that the specific activities of choline in the intra- and extracellular water are identical; since the concentration of choline in the extracellular medium is known the distribution of radioactivity in the steady state allows one to calculate the ratio of choline concentrations.

The steady-state ratios obtained with fresh blood from various donors are shown in Fig. 2. These values suggest an ability to concentrate choline for the following reason: the water content of erythrocytes incubated in isotonic salt solutions at pH 7.4, is 71.7% of the volume (15) so that the concentration of choline in the cell water is 1.39 times the concentration per volume cell. How-

ever, with a transmembrane potential of about 10 mv inside negative, the intracellular and extracellular electrochemical potentials for choline will be equal when the concentration in the intracellular water is 1.4 times the concentration in the external medium. This 1.4 is the reciprocal of the chloride ratio (16). It follows that choline should be at thermodynamic equilibrium when the ratio $\frac{\text{Concentration per volume cells}}{\text{Concentration per volume outside medium}}$ is about one. The data in Fig. 2 indicate therefore that erythrocytes can concentrate choline against an electrochemical gradient.

A concentration ratio larger than one could result from the adsorption of choline to the cell membrane or to intracellular protein. However, when cells equilibrated with $2.5 \mu\text{M}$ ^{14}C -choline are lysed in a hypotonic medium and the

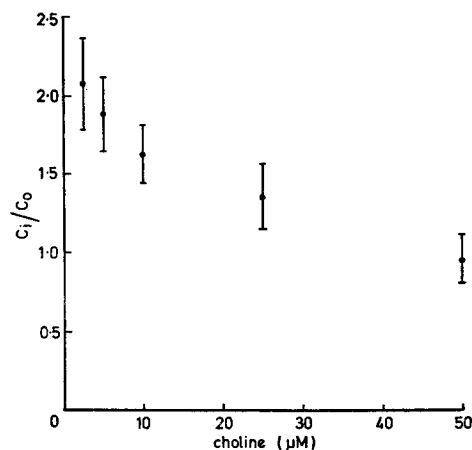


FIGURE 2. The distribution of choline in the steady state as a function of external choline. Means ± 2 SE. Fresh blood obtained from various donors.

$$\frac{C_i}{C_o} = \frac{\text{Concentration per volume packed cells}}{\text{Concentration per volume outside medium}}$$

ghosts spun down there is no indication of radioactivity sticking to the membranes, but it cannot be ruled out that the hemolysis causes a desorption. Also when $2.5 \mu\text{M}$ ^{14}C -choline is added to a 1:4 diluted hemolysate, separation of hemoglobin and choline on a Sephadex (G-75) column does not indicate choline binding. In any case, since the intracellular concentration is calculated from the radioactivity of the supernatant of the TCA precipitate, only adsorption that is reversed by the precipitation procedure could lead to erroneously high values for the cellular concentration of choline. It seems therefore unlikely that adsorption will explain the observed steady-state ratios. The fact that in the absence of external sodium concentration ratios greater than one are no longer observed (cf. Fig. 9) is also evidence arguing against adsorption as an explanation for the accumulation of choline by red cells.

It was not possible to settle whether at higher concentrations of choline in the external medium ($100 \mu\text{M}$ and more) the steady-state ratio remains around one or falls significantly below one. After 16 hr the concentration ratios were

clearly below one, averaging 0.61 at 100 μM choline (four experiments), 0.34 at 200 μM (two experiments), and 0.15 at 500 μM (two experiments). During the following 12 hr the ratios did seem to increase but the net influx was very small and the cells began to hemolyze before satisfactory values for the steady-state ratio could be obtained.

The steady-state values obtained with bank blood were consistently lower than those obtained with fresh blood: the ratio $\frac{\text{Concentration per volume cells}}{\text{Concentration per volume outside medium}}$ was 1.72 at 2.5 μM external choline, 1.45 at 5.0 μM , 1.09 at 25 μM , and 0.82 at 50 μM (each value is based on four experiments). Harris and

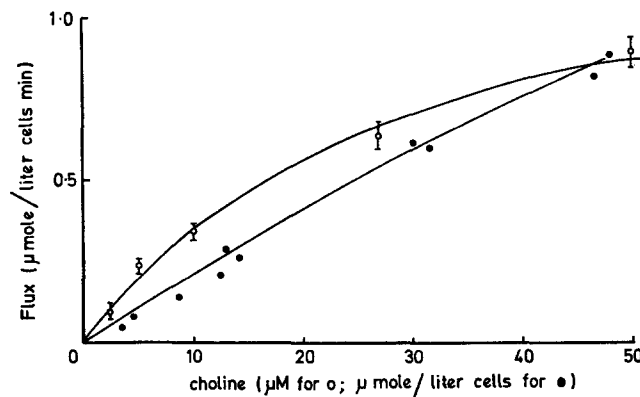


FIGURE 3. Influx (open circles, means ± 2 SE) and efflux (filled circles) during the steady state. The influx curve was calculated assuming Michaelis kinetics and $K_m = 30 \mu\text{M}$, $V_m = 1.4 \mu\text{moles per liter cells per min}$. The efflux curve was calculated from the influx curve and the data in Fig. 2. For the calculated efflux curve and the experimentally measured efflux the units on the abscissa are micromoles per liter cells; for the influx the units are micromolar. All data were obtained with fresh blood from one donor.

Maizels (17) showed that the chloride ratio, $\frac{[\text{Cl}^-]_i}{[\text{Cl}^-]_o}$, is higher with cold stored cells than it is with fresh cells, presumably because cold stored cells have a lower concentration of phosphate ester anions and therefore a lower Donnan potential. It follows that with cold stored cells choline will be at equilibrium when the ratio $\frac{\text{Concentration per volume cells}}{\text{Concentration per volume outside medium}}$ is smaller than one. However, the differences between the steady-state ratios for choline observed with bank blood and those observed with fresh blood are slightly larger than what would be expected from the reported difference in the chloride ratio. Nevertheless, this comparison between fresh blood and bank blood indicates that cold stored cells have lost very little of the ability to concentrate choline. This is surprising but agrees with the observation that overnight incubation at 37°C—which one might expect to alter the metabolic state of the cells con-

siderably—has no apparent effect on the cells' ability to accumulate choline: when the choline concentrations in the external medium are between 2.5 and 10 μM , the steady state is reached after 6 hr and the distribution ratios at that time are the same as those found after 16 and 20 hr.

The Efflux of Choline In the steady state influx equals efflux so that knowledge of the influx and the steady-state distribution for various concentrations of external choline allows calculation of the curve relating the efflux of choline to its intracellular concentration. Fig. 3 indicates that this curve agrees reasonably well with the experimentally measured efflux. The efflux is almost proportional to the intracellular concentration and on first sight compatible with a passive leak. "Pump and leak" systems are frequently postu-

TABLE I
EFFLUX OF ^{14}C -CHOLINE IN THE STEADY STATE

The cells (fresh blood) were loaded with different concentrations of choline by incubating overnight at the concentrations indicated in brackets (micromolar). The values in brackets are also the concentrations of unlabeled choline in the external medium during the efflux experiment. The data are the percentage of radioactivity that has left the cells.

Time	Choline, $\mu\text{moles per liter cells}$				
	4.5 (2.5)	8.8 (5.0)	14.3 (10)	32.5 (25)	48.1 (50)
<i>min</i>					
10	15.2	16.1	16.4	17.1	16.1
30	29.9	32.6	34.4	34.8	33.1
60	43.6	47.2	53.4	55.1	55.4
120	59.2	65.3	68.7	70.3	71.5
240	74.0	79.2	81.4	85.3	87.0
360	83.6	87.7	89.5	92.1	94.4

lated when a substance is actively concentrated and when the steady-state ratio depends on the extracellular concentration of that substance. However, there is evidence that the efflux of choline is not flux through a simple leak. First, the efflux—intracellular choline 12 $\mu\text{moles per liter cells}$ —can be reduced to about 10% of the control value by adding hemicholinium (10^{-4} M) to the external medium. Second, the efflux depends on the concentration of choline in the extracellular fluid (cf. Fig. 5). Third, when the external choline is raised above 50 μM the influx is smaller than what would be expected if such a leak (allowing flux independent of direction) existed.

If the efflux is followed over a longer time (Table I) it appears that the rate constant for the efflux *increases* with the raising of the intracellular choline. A second experiment with blood from the same donor and two experiments with a different blood showed the same pattern and were analyzed by determining the mean rate constants for the fluxes at various choline concentrations follow-

ing the approach of Creese, Neil, and Stephenson (13) as described in the section on methods.

This analysis indicates that red cells are nonhomogeneous with respect to their choline permeability. This is not surprising considering the heterogeneity of erythrocytes with regard to sodium and potassium content and transport properties (16). When fluxes based on the mean rate constant are used this leads to a maximum flux which is 20–30% lower than that calculated from the initial rates; the K_m is virtually the same.

The reciprocals of the fluxes that obtain when the mean rate constant is multiplied by the intracellular concentration of choline, were plotted in Fig. 4

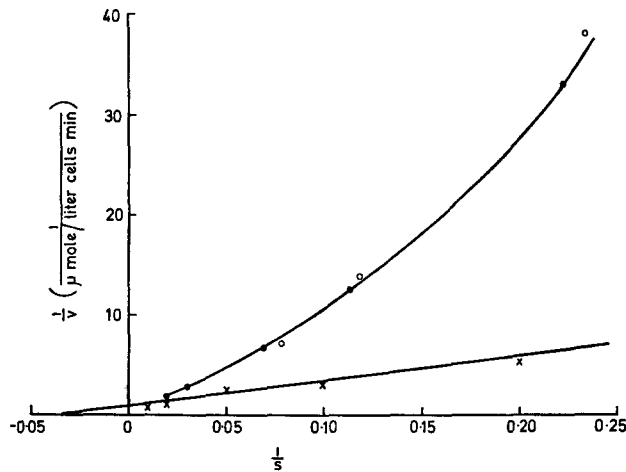


FIGURE 4. Lineweaver-Burk plots of efflux (open and filled circles, two experiments with fresh blood from one donor) and influx (crosses, one experiment, fresh blood from the same donor) during the steady state. All flux values were calculated with mean rate constants. For efflux S = micromoles per liter cells, for influx S is micromolar.

against the reciprocals of the cellular concentration. The curve indicates deviation from Michaelis-Menten kinetics and is similar to curves obtained with enzymes that show “substrate activation” (18). However, an alternative explanation for the efflux kinetics will be considered in the discussion.

A comparison of this efflux curve with the straight line fitting influx data (this time also calculated using mean rate constants) suggests that as the concentration of choline is increased the slope of the efflux curve approaches that of the influx curve. Also, it seems that the intercept of the two curves with the ordinate might be the same, which would mean that the maximum rates for influx and efflux are the same. It is, of course, desirable to measure efflux at higher concentrations of choline but, as pointed out earlier, cells usually deteriorate before the intracellular choline rises appreciably above 50 μ mole per liter cells.

Exchange Flux Phenomenon The initial rate of ^{14}C -choline influx into cells preequilibrated with a choline-free buffer is considerably smaller than the influx into cells preloaded with unlabeled choline (Fig. 1). After 6 hr, when cells incubated at 2.5 and 5.0 μM ^{14}C -choline had reached a steady state with respect to the labeled choline the intracellular concentrations of label in the two groups were identical. Reduced backflux of ^{14}C -choline from the cells preloaded with unlabeled choline cannot account for the difference in influx rates during the initial 10 min and it seems therefore that the influx is affected by the intracellular concentration of choline. To see whether the efflux of ^{14}C -choline is similarly affected by the concentration of unlabeled choline in the external medium, cells were loaded overnight with labeled choline and then placed into large volumes of choline-free and choline-containing media.

TABLE II
EFFLUX ($\mu\text{MOLES PER LITER}$
CELLS PER MIN) DURING STEADY STATE (V_s)
AND INTO CHOLINE FREE MEDIA (V_o)

The values in brackets under V_s have the same meaning as in Table I. Bank blood.

Choline	V_s	V_o	$\frac{V_s}{V_o}$
<i>$\mu\text{moles per liter cells}$</i>			
4.6	0.081 (2.5)	0.066	1.22
8.7	0.143 (5.0)	0.088	1.62
18.0	0.283 (10)	0.143	1.97
32.9	0.624 (25)	0.243	2.56
53.5	0.963 (50)	0.309	3.11

The hematocrit was less than 5% so that the backflux of label is negligible in these experiments. The results (Table II) reveal again that the flux of labeled choline is higher when the medium on the other side of the membrane contains choline. Also the ratio $\frac{V_s}{V_o}$, when V_s is the flux during the steady state and V_o the flux into zero choline, becomes larger as the concentration of choline is increased. The dependence of ^{14}C -choline efflux on external choline is therefore rather similar to the dependence of influx on internal choline.

More information on the exchange flux phenomenon was obtained by loading cells to 53 μmoles choline per liter cells and incubating aliquots in different solutions containing from zero to 100 μM choline (Fig. 5). As the external choline concentration is raised from zero to 50 μM the effect on efflux is pronounced but it becomes small thereafter.

The dependence of choline influx on internal choline can be studied in a similar way by preequilibrating cells with various concentrations of unlabeled

choline prior to measuring the influx from a solution containing $50 \mu\text{M}$ choline. When influx is plotted against the choline concentration of the preequilibration medium one obtains a curve rather similar to the efflux curve shown in Fig. 5. However, there is no similarity between influx and efflux curve if the influx is plotted against the actual intracellular concentration of choline. This can be interpreted in the following way. Preincubation in say $10 \mu\text{M}$ choline raises the intracellular choline to about $17 \mu\text{moles}$ per liter cells. However, this intracellular concentration of choline stimulates the influx of labeled choline only to the same extent that efflux is stimulated by an external concentration of $10 \mu\text{M}$. On the other hand, the influx associated with $10 \mu\text{M}$ choline outside is equal to the efflux associated with an intracellular concentration of $17 \mu\text{moles}$ per liter cells. One might therefore argue that the influx of labeled

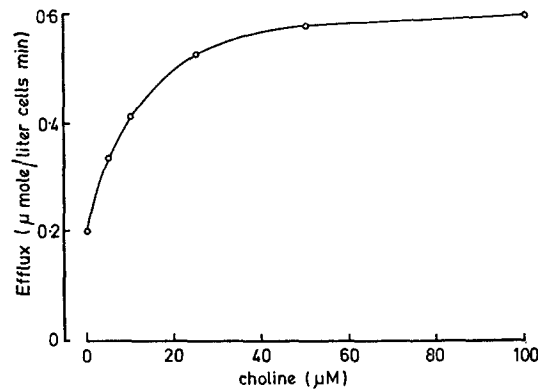


FIGURE 5. Efflux (initial rates) from cells containing $53 \mu\text{moles}$ choline per liter cells into media containing different concentrations of unlabeled choline. Bank blood was used; the curve was drawn by eye.

choline is increased by an *efflux* of unlabeled choline rather than by the *presence* of choline inside. However, such a hypothesis should be tested by experiments using simultaneously ^{14}C -choline and ^3H -labeled choline or other quarternary ammonium ions.

Effect of Temperature The experiment shown in Table III was done to see whether lowering the temperature would affect the exchange flux phenomenon. Reduction of the temperature from 37° to 10°C slows both fluxes, that into choline-free solutions and that into choline-containing solutions to the same extent; the apparent activation energy is about 14 cal/mole .

Effect of Ouabain In experiments lasting up to 4 hr $5 \times 10^{-5} \text{ M}$ ouabain in the external medium had no significant effect on influx or efflux of choline.

The Role of Sodium Cells incubated in a sodium-free medium have apparently lost the ability to concentrate choline but can regain it if transferred

to a sodium-containing medium (Fig. 6). If cells that have concentrated choline in a sodium-containing medium are transferred to a sodium-free medium a net efflux of choline occurs until the ratio of electrochemical potentials is about one. It seems that erythrocytes can concentrate choline and maintain a choline gradient only in the presence of external sodium.

The data in Fig. 7 show the effects of gradually replacing sodium, this time by lithium. It appears that the concentration of sodium affects the initial rate of influx and the steady state to a similar extent. No attempt has been made to establish with these data the exact relationship between sodium, choline influx, and steady-state value. It seems, however, that raising the external

TABLE III
EFFLUX (μ MOLES PER LITER CELLS) AT 10° AND 37°
INTO ZERO, 50, AND 1000 μ M CHOLINE

The internal concentration of 14 C-choline was 53.5 μ moles per liter cells. Fresh blood. The table gives values for the efflux during 10, 30, and 60 min because at 10° the efflux is very small resulting in less accurate values especially for the 10 min period. The data show that the efflux at 10°C increases when raising the extracellular choline from zero to 50 μ M and to 1000 μ M, indicating an exchange flux which is similar to that observed at 37°C.

Extracellular choline	10 min	30 min	60 min	Temperature
μ M				°C
0.0	3.09	6.55	11.0	37
	0.64	0.75	1.41	10
50	9.63	18.4	30.8	37
	0.85	2.2	2.89	10
1000	11.2	22.1	33.1	37
	0.91	2.41	3.75	10

sodium from zero to 70 mM increases the choline influx as much as raising the sodium from 70 to 140 mM; in other words, the relationship might be linear.

An alternative explanation for these data is that both potassium and lithium have a very similar inhibitory effect on the choline transport mechanism. However, these two ions differ considerably in their effect on enzymes (e.g. Na^+ - K^+ ATPase) and other transport mechanisms (e.g. membrane conductance in excitable tissues). It seems therefore more likely that the inhibition observed on replacing sodium by potassium or lithium is attributable to the absence of sodium.

It is possible to argue that what appears to be a sodium-dependent choline pump is in fact a sodium-dependent adsorption of choline to the erythrocyte membrane. This possibility is ruled out by the experiment in Fig. 8. Aliquots of cells that had concentrated choline during the previous 16 hr were trans-

ferred to a sodium buffer, a sodium-free potassium buffer, and a sodium-free potassium buffer containing 10^{-3} M hemicholinium. Independent experiments on the effect of hemicholinium on choline influx and efflux have shown that at the low choline concentrations used— $2 \mu\text{M}$ in the external media—the carrier-mediated influx and efflux of choline are effectively blocked by the high concentration of hemicholinium. With choline movement blocked in this way the removal of external sodium has no effect on the intracellular concentration of choline. It is difficult to see why hemicholinium should prevent the desorption

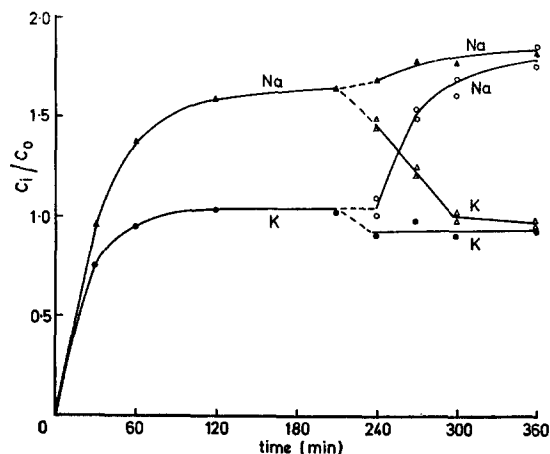


FIGURE 6. The effect of external sodium on the choline transport system. Cells incubated for 200 min in a 151 mM Na buffer (filled triangles) were washed and aliquots transferred to another Na buffer (filled triangles) as well as to a sodium-free buffer containing 151 mM K (open triangles). Similarly, cells first incubated in the absence of sodium (filled circles) were transferred to a Na (open circles) and to K (filled circles) buffers. All solutions contained $2.5 \mu\text{M}$ ^{14}C -choline. The experiment was done with fresh blood which had not been preequilibrated overnight with a known concentration of choline.

$$\frac{C_i}{C_o} = \frac{\text{Radioactivity per volume packed cells}}{\text{Radioactivity per volume outside medium}}$$

of choline from the cell membrane and it seems that external sodium is necessary for the functioning of a true choline pump.

The experiment in Fig. 9 shows the effect of replacing sodium by potassium or lithium on the steady-state values for two concentrations of choline. This particular batch of cells (bank blood) gave low concentration ratios: 1.6 with $2 \mu\text{M}$ and 0.98 with $15 \mu\text{M}$ choline. As the external sodium is reduced the steady-state ratios fall and approach each other until—at zero sodium—they are independent of the choline concentration; this is consistent with the idea that in the absence of external sodium the erythrocytes are unable to concentrate choline. When sodium is replaced by potassium the steady-state ratios—

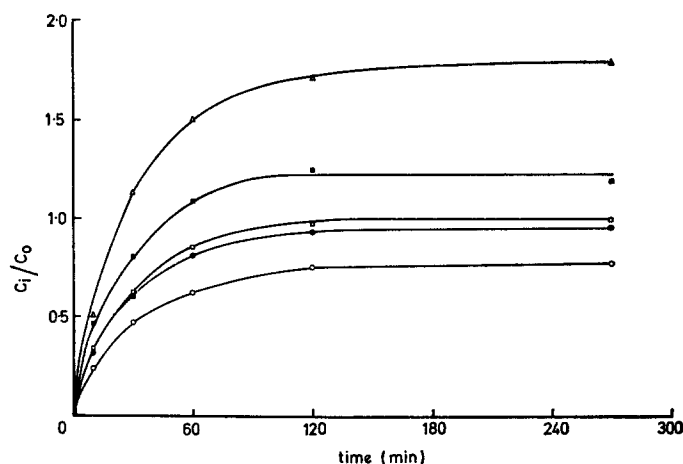


FIGURE 7. Effect of replacing sodium by lithium on influx and steady-state distribution of choline. The concentration of ^{14}C -choline in the external medium was $2.5 \mu\text{M}$. Bank blood, not preequilibrated. $\frac{C_i}{C_o}$ as in Fig. 6. Na (mM) in the external medium 140 (open triangles), 70 (filled squares), 25 (open squares), 5 (filled circles), zero (open circles).

for both choline concentrations—are 0.84 while in the lithium medium the corresponding values are only around 0.63. It is conceivable that the carrier can interact not only with sodium but also with potassium and lithium; in this case the properties of the transport system in the two sodium-free media might be slightly different. However, it is also possible that the transmembrane potential is to some extent affected by the external cation so that the concentration ratio at which choline is at thermodynamic equilibrium is different in the two media. No attempt was made to differentiate between these two possibilities.

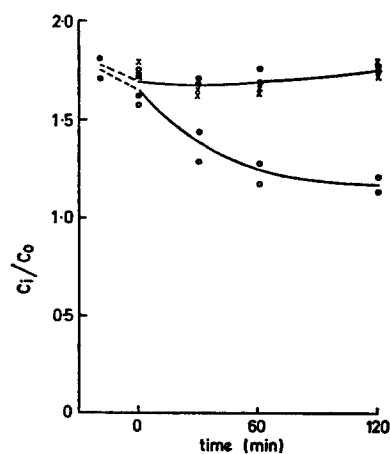


FIGURE 8. The effect of hemicholinium on the sodium-dependent ability of erythrocytes to concentrate choline. Cells incubated overnight in a Na buffer containing $2 \mu\text{M}$ ^{14}C -choline (filled circles) were washed and transferred to another Na buffer (filled circles), to a Na-free K buffer (open circles), and to a Na-free K buffer containing 10^{-8}M hemicholinium (crosses); all solutions contained $2 \mu\text{M}$ ^{14}C -choline. The experiment was done with fresh blood. $\frac{C_i}{C_o}$ as in Fig. 6.

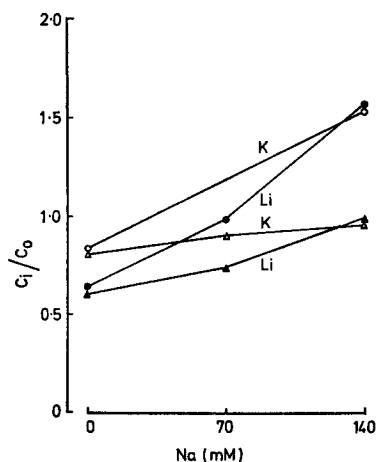


FIGURE 9. The effect of replacing sodium by lithium (filled circles, filled triangles) or potassium (open circles, open triangles) on the steady-state distribution of ^{14}C -choline when the external concentrations are $2\ \mu\text{M}$ (open circles, filled circles) and $15\ \mu\text{M}$ (open triangles, filled triangles) choline. The steady-state distribution was determined after 16 and 20 hr; there was no change in intracellular activity during these 4 hr. Bank blood was used. $\frac{C_i}{C_o}$ as in Fig. 6.

The effects of removing the external sodium raise the question whether the energy necessary to concentrate choline might be derived from the electrochemical gradient of sodium. To study the effect of intracellular sodium, aliquots of stored blood were placed in a sodium-free (150 mM K) and full sodium (150 mM Na) buffer and for 10 days mixed in slowly rotating bottles in the cold; the outside solutions were changed every day. Measuring the uptake of choline by these cells at 37°C (Fig. 10) revealed that in the presence of external sodium erythrocytes will concentrate choline to the same extent whether the internal sodium is low or high. In the absence of external sodium the choline uptake is reduced but again independent of the intracellular sodium.

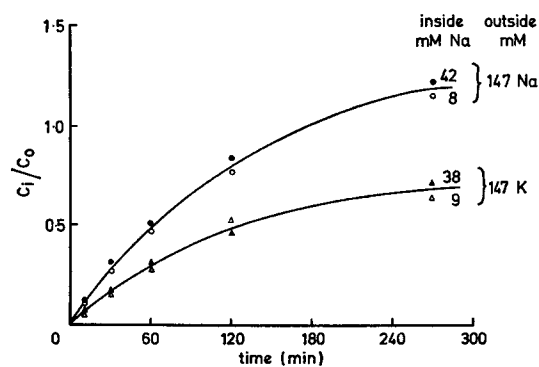


FIGURE 10. The effect of internal sodium on choline transport. High sodium (filled circles, filled triangles) and low sodium cells (open circles, open triangles) were incubated in a high sodium (open circles, filled circles) and a sodium-free (open triangles, filled triangles) medium. Intracellular sodium was determined at the end of the experiment with a flame spectrophotometer and is expressed as concentration in the intracellular water. The concentration of ^{14}C -choline was $2.5\ \mu\text{M}$ in all media. $\frac{C_i}{C_o}$ as in Fig. 6.

Though these cells are not yet in the steady state they are rather close to it and it seems that in the presence as well as in the absence of external sodium the steady state is *not* a function of the electrochemical gradient of sodium. It follows that in contrast to the influx the efflux is not activated by sodium.

The question of the energy source of the choline pump has not been settled. In experiments lasting up to 20 hr the ability of the cells to concentrate choline was not affected by eliminating glucose from the incubation medium. Experiments involving longer incubation periods are usually complicated by hemolysis. However, in one satisfactory experiment cells were incubated for 36 hr without metabolizable substrate and in the presence of 10 mM inosine. All solutions contained 5 mM phosphate. Cells incubated with inosine did concentrate choline ($\frac{C_i}{C_o} = 1.7$ with 2.5 μM choline in the external medium) while cells incubated without metabolizable substrate had apparently lost the ability to do so ($\frac{C_i}{C_o} = 1.12$). It seems unlikely that inosine produced this effect by protecting cells against becoming leaky to choline because with aliquots of these cells incubated in 5 and 20 μM choline the concentration ratios at 4 hr were 1.18 and 0.68 in the presence of inosine but 0.80 and 0.38 in the substrate-free medium. If cells incubated without substrate were leaky to choline they should show concentration ratios close to one at high as well as at low concentrations of choline.

DISCUSSION

The present experiments suggest a transport system in erythrocytes that will concentrate choline because at concentrations below 50 μM the apparent affinity constant of the influx carrier is higher than that of the efflux carrier. In this concentration range the influx agrees reasonably well with Michaelis-Menten kinetics. The kinetics of the efflux in the steady state are more complicated; the possibility that it represents flux through a passive leak can be clearly ruled out because the efflux can be inhibited by hemicholinium and shows an exchange flux phenomenon.

The kinetics of carrier-mediated transport have recently been analyzed by Silverman and Goresky (19). In this model the interaction between carrier and substrate follows Michaelis kinetics and the carrier is present in two forms differing in their affinity for the substrate. The transformation of carrier from one form into the other depends on asymmetric metabolic reactions.

The transport model of Silverman and Goresky can to some extent be applied to the choline transport system described here. The model predicts a relationship between steady-state distribution and the external concentration of the substrate which is of the same type as that shown in Fig. 2. Furthermore, the fact that the kinetics of the efflux in the steady state are approximately

first order (Fig. 3) is consistent with the model. It is therefore not necessary to invoke special interactions between choline and the efflux carrier—such as substrate activation—to explain the efflux kinetics.

Silverman and Goresky (19) point out that in a carrier-mediated transport system with a moderate capacity for concentration the kinetics of the influx might reveal the presence of both carrier forms, that with a high and that with a low affinity for the substrate. The results presented here do not allow a decision as to whether there are two influx components because influx in the steady state could be studied only at low choline concentrations. Askari (3) has measured the influx of choline into erythrocytes at higher choline concentrations but in these experiments the intracellular concentration of choline was unknown. Since the influx of labeled choline is *not* independent of the efflux of unlabeled choline the interpretation of results obtained in this way is complicated.

In any case, the model suggested by Silverman and Goresky cannot be rigorously applied to the choline transport system because it does not allow for an exchange flux in a situation in which the affinity of the carrier is the same on the two sides of the membrane. In other words, only transport systems capable of concentrating a substrate should show an exchange flux phenomenon. However, preliminary experiments with ghosts indicate clearly that with the choline carrier the exchange flux phenomenon is not restricted to an asymmetrical and concentrating transport system. Similar evidence has been obtained with the facilitated transport of sugar across the red cell membrane (20, 21).

When the external sodium is removed the inward movement of choline by the carrier is reduced and the cell's ability to concentrate choline is apparently lost. With transport systems concentrating amino acids (22) and sugars (23) the effect of removing external sodium is to lower the affinity of the carrier for the substrate. Whether the interaction between sodium and the choline influx carrier is of a similar kind has not been investigated.

Most active transport systems are sodium-dependent and hypotheses interpreting this dependence often imply that the uphill movement of substrate is coupled to the downhill movement of sodium. These hypotheses predict furthermore that the direction of the pump can be reversed by reversing the sodium gradient. Experimental evidence concerning the mechanism of Na activation has been reviewed by Heinz (24); recent data on the role of sodium in the amino acid transport by red cells have been published by Wheeler and Christensen (25). The present data are not consistent with the idea that the movement of choline is directly coupled to the electrochemical gradient of sodium. However, it cannot be ruled out that activation of influx carrier involves passage of sodium into the cell.

It was pointed out earlier that the exchange flux observed here cannot be attributed to competitive inhibition of the ^{14}C -choline backflux by the un-

labeled choline; it is also not explained by a model of the type suggested by Silverman and Goresky (19). The usual interpretation of exchange flux invokes a mobile carrier and postulates that the loaded carrier moves faster across the membrane than the unloaded one. The data presented here are, of course, compatible with such a theory, but exchange flux can also result from a mechanism that does not involve a moving carrier (26).

The maximum rate of "carrier-mediated" choline transport is very small when compared with the rate at which sodium and potassium cross the red cell membrane. It is therefore justifiable to consider choline a "nonpenetrating" cation when it is used to replace sodium and potassium in experiments studying the properties of cell membranes. The small "passive" permeability of the red cell membrane to choline that becomes evident when higher concentrations of choline are used has been described and discussed by Askari (3).

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