Uptake of Some Quaternary Ammonium Ions by Human Erythrocytes

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ABSTRACT In many biophysical studies on erythrocytes some quaternary ammonium ions are used as replacements for Na^+ and K^+ of the physiological solutions. The object of this work was to study the possible uptake of quaternary ammonium ions by erythrocytes. Uptake of C^{14}–choline chloride and C^{14}–tetramethylammonium chloride by human erythrocytes was proved. It was shown that the compounds were neither incorporated into phospholipids of the cell nor converted to any other metabolites. Studies of uptake as a function of time, at several external concentrations of choline and tetramethylammonium, showed that within the first 4 hours uptake was a linear function of time regardless of the external concentration of the quaternary ammonium ions. The effects of various external concentrations of choline and tetramethylammonium ions on the rate of uptake by the cells were studied. The results showed the presence of two distinct mechanisms for the uptake of choline: one, a facilitated uptake mechanism which becomes saturated at low external concentrations of the ion; the other, a simple diffusion mechanism in which the rate of uptake is proportional to concentration. For the facilitated part of the uptake the external choline concentration at which half-maximum rate was obtained was found to be 0.02 mM. Although the kinetic studies with tetramethylammonium ion were not as extensive as those with choline, they did suggest the presence of similar mechanisms for the uptake of both ions. Tetramethylammonium and tetraethylammonium ions were shown to be competitive inhibitors of the facilitated choline uptake.

INTRODUCTION

Biochemical and nutritional studies long ago suggested that at least some of the cells within the gastrointestinal tract are permeable to some quaternary ammonium ions. For example, when choline was fed to rats its participation in the process of transmethylation within tissues was shown (1). More recently the uptake of several quaternary ammonium ions by intestinal cells, skeletal muscle, and parts of the central nervous system has clearly been demonstrated (2–4). It has also been known for some time that a variety of quaternary ammonium ions are rapidly secreted by the kidney (5). In spite of these facts, in

many studies on the biophysics of various cellular membranes, choline and other permanently charged quaternary ammonium ions are used as "non-penetrating" cations. The justification for such uses is based on the assumption that the rate of penetration of a quaternary ammonium ion is much lower than those of the physiologically important cations such as Na^+ and K^+. However, sufficient data on the quantitative aspects of quaternary ammonium ion uptake by various tissues are not available.

A few attempts to measure the uptake of a quaternary ammonium ion by a tissue have been made in the past. Most have been limited to the demonstration of possible uptake by a tissue and include no quantitative data on the kinetics of the process (2, 3, 6–8). Quantitative measurements of choline uptake by skeletal muscle (9) and the uptake of some quaternary ammonium ions by choroid plexus (4) have been reported. Although choline and other simple quaternary ammonium ions have been used extensively in studies on the alkali cation permeability of human erythrocytes (e.g., 10, 11), no data on the possible uptake of these quaternary ammonium ions by erythrocytes are available. This study was, therefore, undertaken to test such a possibility. Uptake of choline and tetramethylammonium ions by erythrocytes was proved and kinetics of uptake was studied.

MATERIALS AND METHODS

Me-C^{14}–choline chloride, 35 mc/mmole, was obtained from Nuclear Chicago, Des Plaines, Illinois; and C^{14}–methyl iodide, 10 mc/mmole, was purchased from New England Nuclear Corp., Boston, Massachusetts.

C^{14}–tetramethylammonium chloride was synthesized by modification of a method for the synthesis of C^{14}-choline (12). C^{14}–methyl iodide was reacted with trimethylamine in an atmosphere of nitrogen. The crystalline C^{14}–tetramethylammonium iodide was converted to the chloride salt by treatment with silver chloride. The white crystalline solid was then dissolved in distilled water and stored frozen.

Procedure for the Uptake Studies

Fresh blood (20 ml) was withdrawn, mixed with heparin (200 USP units), and centrifuged at 1000 *g* for 10 minutes. Plasma and buffy coat were removed and erythrocytes were then washed four times with 40 ml of a solution which had the following composition: Na^+, 145 mM; K^+, 5.3 mM; Ca^{2+}, 1.3 mM; Mg^{2+}, 1.3 mM; Cl^-, 137 mM; phosphate, 8.6 mM; sulfate, 1.3 mM; glucose, 5 mM (pH, 7.4). After each washing, mixing of the cells with the wash solution and centrifugation at 1000 *g* for 10 minutes; the supernatant and one-third of the top layer of the cells were then removed and discarded. Strict adherence to this washing procedure is essential if erythrocytes free from white cells and platelets are to be obtained. Washed cells (0.1 ml) were then added to 1.5 ml of the incubation medium, which had already been brought to 37°C. The incubation medium had the same composition as the wash solution with the following exception: whenever a quaternary ammonium chloride was added,

the equivalent amount of NaCl was omitted. The cell suspension was placed in a water bath at 37°C and shaken for an appropriate period of time; 1 ml of the mixture was then withdrawn and mixed with 9 ml of ice cold wash solution in a 10 ml narrow bottom centrifuge tube calibrated to 0.1 ml in 0.01 ml steps. The tube was centrifuged at 2500 *g* for 3 minutes and the supernatant withdrawn. The cells were washed three more times with 10 ml portions of cold wash solution. Preliminary experiments showed that, with the levels of radioactivity used in these studies, the supernatant from the last washing did not contain any activity over the background. Furthermore, if the cells were washed more than three times their radioactivity did not decrease appreciably. The volume of the packed cells was recorded after each centrifugation. No changes in cell volume, after the incubation and washings, were observed. (It should be noted that volume changes of perhaps up to 15% would not have been detected by the method used. However, swelling or shrinking did not affect the uptake calculations, since these were based on the hemoglobin content of the cells.) Solid samples were prepared from the cells and counted.

Sample Preparation and Counting

To the washed cells, which had been packed in the calibrated centrifuge tube, a solution of 15% choline chloride or tetramethylammonium chloride in 0.1 N HCl was added to bring the total volume to 1 ml. The cells were then thoroughly mixed with the solution; 0.1 ml of the mixture was withdrawn and used for hemoglobin determination by the acid hematin method, and 0.7 ml was pipetted into a weighed glass planchet (1 inch in diameter) and left at room temperature for 48 hours. The sample was then further dried under an infrared lamp for 15 minutes and immediately thereafter placed in a gas flow G. M. detector. After counting, the planchet was again weighed. Similar samples were also prepared using fresh packed cells which did not contain any radioactivity. Before drying, known amounts of labeled quaternary ions were added to the samples. These were then dried and counted as before. By comparison of the counts of the standard samples with those of samples obtained from labeled cells, the amounts of quaternary ion uptake per quantity of hemoglobin were calculated. These were then converted to uptake per volume of cells on the basis of 6 mM hemoglobin content.

It should be noted that in the counting of samples containing quaternary ammonium compounds and in the quantitative comparison of the counts special problems are involved. Since the compounds are extremely hygroscopic and in some cases heat labile (13), preparation of infinitely thin samples is not practical. The simple procedure outlined above for the preparation of solid samples overcame all the difficulties and was sensitive enough for the levels of counts obtained in these studies. The addition of the acidified solution of nonradioactive quaternary compounds to the samples served two purposes: It prevented any possible loss of activity due to decomposition during the long drying period, and it also increased the weight of the sample over the critical level necessary to obtain an infinitely thick sample. This level was determined experimentally. The slow drying was necessary in order to obtain a smooth uniform surface in every sample. The final drying with the infrared lamp did not damage the uniform geometry of the sample. The use of the gas

flow counter had the advantage that the sample was kept dry throughout the counting period No changes in the weight of a sample could be detected during 2 hours of counting.

A Nuclear Chicago model D-47 gas flow counter with a thin window and a Nuclear Chicago 186 scaler were used for counting. The counting time for each sample was chosen to equalize the inherent errors of the counting procedure for all the samples. The standard deviation of each count was no more than 3%.

Column and Paper Chromatography

A modification of the method of Christianson *et al.* (14) was used for column chromatography. A column of Dowex 50 W × 8, 200 to 400 mesh, 40 cm high and of 1.5 cm in diameter, was set up and washed with 2 liters of 1.5 N HCl. Solution of the test compounds in 1.5 N HCl was applied and eluted with the same acid solution, and 2 ml fractions were collected. When quantitative comparisons of the radioactivities of the fractions were desired, infinitely thick samples were prepared by a procedure similar to that described above. Choline could be separated cleanly from aminoethanol, monomethylaminoethanol, and dimethylaminoethanol. Also, tetramethylammonium chloride could be separated from the other methylamines on this column.

Paper chromatographic separation of choline and trimethylamine was carried out according to Tolbert *et al.* (15). Spots of amines on paper were detected by the modified Dragendorff reagent (16), and the amines in solution were detected or measured as periodides (17).

Test of Purity of C^{14}-Choline and C^{14}-Tetramethylammonium Chloride

To learn whether quantitative studies based on the measurement of cell radioactivity were feasible at all, it was important to determine whether any radioactive impurities were present in the labeled compounds and to measure the extent of these. The instability of C^{14}-choline and the problems associated with its use are well known (15, 18). The most likely impurity in a choline sample is trimethylamine, which could be produced from the decomposition of choline. Other possible impurities are monomethyl- and dimethylaminoethanol. The possible impurities in C^{14}-tetramethylammonium chloride are other methylamines.

A sample of C^{14}-choline chloride was mixed with nonradioactive trimethylamine hydrochloride and chromatographed on paper. The scan of the paper showed only one intense radioactive peak with an R_f corresponding to that of choline. By eluting the choline and the trimethylamine spots and counting the eluates it was calculated that the purity of C^{14}-choline in respect to contamination with C^{14}-trimethylamine was over 99.4%. Labeled choline chloride was also chromatographed on the ion exchange column. Aliquots of all the fractions containing choline, and the fractions containing aminoethanol, methylaminoethanol, and dimethylaminoethanol were counted. Calculations based on these counts showed that the purity of C^{14}-choline, in respect to contamination with the aminoethanols, was over 99.9%. A similar procedure used with a sample of C^{14}-tetramethylammonium chloride proved the radiopurity of this compound, in respect to contamination with the other methylamines, to be over 99.1%.

RESULTS AND DISCUSSION

Proof of Uptake of Choline and Tetramethylammonium Ions

Preliminary experiments on the uptake of C^{14}-choline and C^{14}-tetramethylammonium ions showed that a very small fraction of the total activity of the incubation medium was taken up by the cells. Even though the most likely radioactive impurities were shown to be present, if at all, in very small amounts, it was not permissible to correlate the measured radioactivity of the cells with the uptake of the quaternaries from the medium without further studies. Two possibilities had to be ruled out: (*a*) the presence of other radioactive impurities which could easily be taken up by the cells, (*b*) the conversion of the quaternaries to other compounds, such as trimethylamine, prior to the uptake. Furthermore, it was important to know whether the quaternary taken up by the cell remained in the original form or was converted to another compound within the cell. This knowledge is essential especially when the release of quaternaries from the cells, not dealt with in this paper, is studied. In the case of choline the possibility of incorporation into phospholipids had to be considered. Although careful studies have shown that no synthesis of phospholipids occurs in erythrocytes (19), there are contrary reports (20) and the problem had to be reinvestigated.

Erythrocytes were incubated in a solution containing 0.11 mM C^{14}–choline chloride for 2 hours and then washed according to the procedure described under "Methods." Several incubation tubes were used so that 2 ml of the washed packed cells were obtained. Determination of the activity of an aliquot of the cells showed that 0.13% of the total activity of the medium was taken up by the cells. The remaining cells were then extracted with methanol-chloroform according to Dawson *et al.* (21), and 97% of the cellular activity was recovered in the extract. No activity could be detected in the precipitated proteins. The extract was shaken with 0.9% NaCl to remove all the nonlipid materials. The organic layer containing the phospholipids did not show any activity; counting an aliquot of the aqueous portion showed that 95% of the activity of the cells had been recovered in this fraction. The remainder of the aqueous extract was dried in vacuum. The solids were extracted with six 1 ml portions of absolute ethanol and the ethanol solution was filtered and dried in vacuum. The solids were again extracted with 0.5 ml of absolute ethanol, and 82% of the original activity of the cells was found in this solution. When an amount of C^{14}-choline equivalent to the activity found in the cells in the above experiment was mixed with nonradioactive erythrocytes and taken through the same procedure, 84% of the activity was recovered in the final ethanol solution. Nonradioactive choline and trimethylamine were dissolved in an aliquot of the ethanol solution obtained from the radioactive cells and

chromatographed on paper and on the ion exchange column. In all cases, all the radioactivity remained with the choline spot or the fractions (Fig. 1). When similar experiments were performed with C^{14}–tetramethylammonium chloride, 0.08% of the total activity of the medium was taken up by the cells and 86% of the activity of the labeled cells was recovered in the final ethanol extract, compared with 85% recovery in a control experiment. All the radioactivity of the ethanol solution remained with tetramethylammonium when the solution and tetramethylammonium were mixed and chromatographed on ion exchange column.

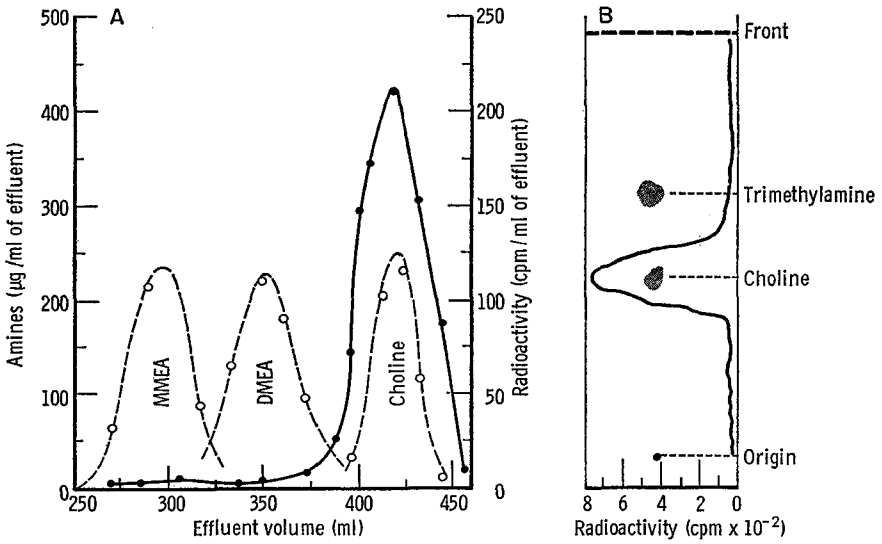

FIGURE 1. Identification of the radioactive material isolated from cells which were incubated in a medium containing [^{14}C] choline. A, column chromatography of a mixture of the labeled material (solid circles) with monomethylaminoethanol (MMEA), dimethylaminoethanol (DMEA), and choline (open circles). B, paper chromatography of a mixture of the labeled material with trimethylamine and choline. Record of the scan of the paper for radioactivity has been superimposed on the paper.

The results of the experiments presented clearly demonstrate that (*a*) choline and tetramethylammonium are taken up by human erythrocytes; (*b*) they are neither incorporated into phospholipids of the cell nor converted to any other metabolites; (*c*) all the radioactivity of the cells incubated with C^{14}-choline or C^{14}-tetramethylammonium can be accounted for by the two compounds, and, therefore, quantitative measurement of uptake based on the determination of cell radioactivity is possible.

To investigate the possibility of the binding of a large amount of choline to the cell membrane, some washed labeled cells, prepared as in the previous experiment, were hemolyzed in 6 volumes of cold water. A portion of the hemol-

ysate was centrifuged at 20,000 *g* for 10 minutes. Radioactivities of equal aliquots of the whole hemolysate and the stroma-free supernatant were measured. No difference between the two values was found. These results suggest that the measured uptake of choline by the intact cell is not a reflection of the tight binding of the compound to the cell membrane.

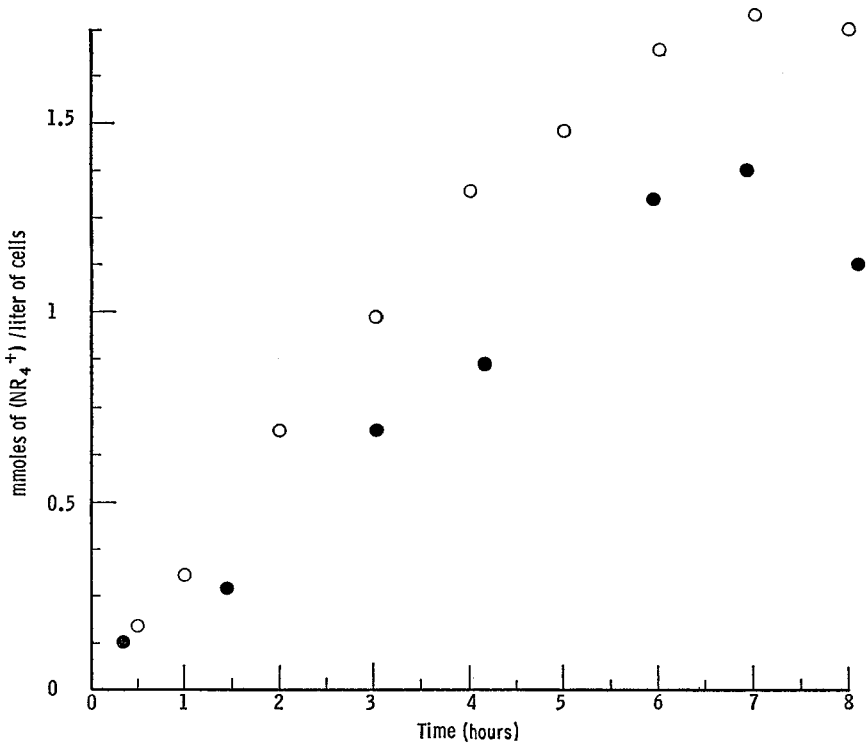

FIGURE 2. Uptake of choline (open circles) and tetramethylammonium (solid circles) by human erythrocytes as a function of time. The external concentration of each compound was 50 mM.

Kinetics of Uptake of Choline and Tetramethylammonium Ions

In order to compare the rates of uptake at various external concentrations it was essential to choose incubation periods during which uptake was a linear function of time. Fig. 2 shows the uptake of choline and tetramethylammonium by erythrocytes as a function of time. The external concentration of each compound in these experiments was 50 mM. It is evident that within the first 4 hours the amount of uptake is a linear function of time. Thereafter a decrease in the rate of uptake is observed. When similar experiments were performed with four other external concentrations of choline (0.01 mM, 0.5 mM, 10 mM, and 120 mM), curves very much like the one presented in Fig. 2, with decrease in the rate after the first 4 to 6 hours, were obtained. These experiments show

that a period of 4 hours is suitable for rate studies regardless of the external concentration of the quaternaries. The cause of the deviation of the uptake vs. time plot from linearity after 4 to 6 hours is not known. Perhaps with longer periods of incubation a general deterioration of the cells occurs. In this con-

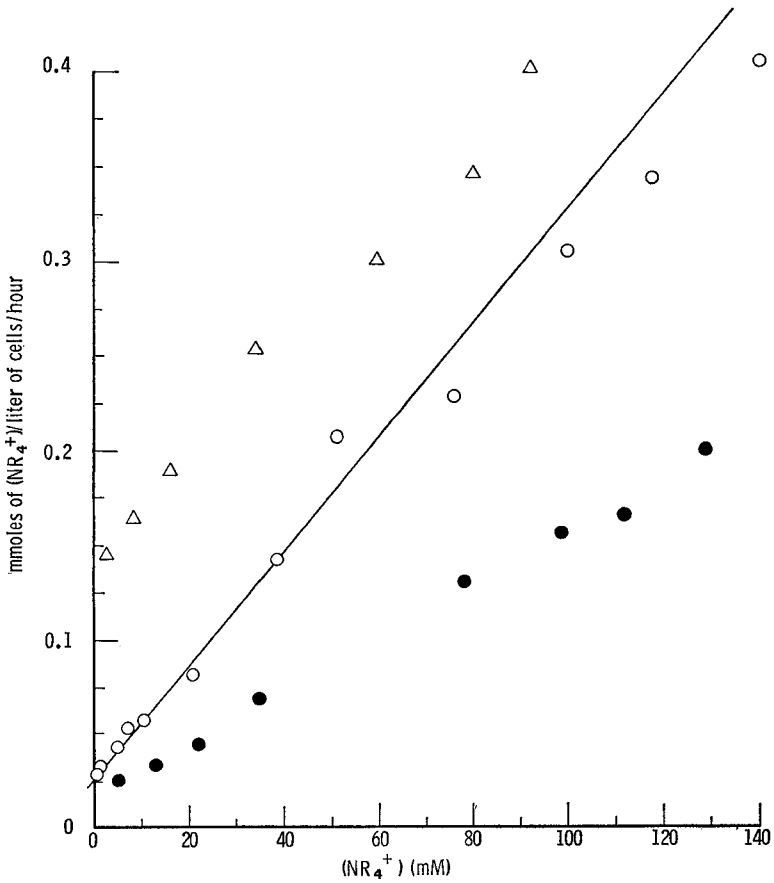

FIGURE 3. Effect of varying external concentrations of choline and tetramethylammonium on the rates of uptake of the ions by erythrocytes. Open circles, choline uptake by the cells of donor A; triangles, choline uptake by the cells of donor B; solid circles, tetramethylammonium uptake by the cells of donor A. The points represent experimental values, and the line represents the equation v = [0.014 (choline)]/[0.02 + (choline)] + 0.003 (choline), in which v is the rate of uptake of choline.

nection it should be mentioned that after 6 to 8 hours of incubation a considerable amount of hemolysis, evidenced by the pink color of the supernatant after centrifugation, was observed. Another likely interpretation is that the time required to approach a constant intracellular concentration of quaternary ions is independent of the external concentration of the ions. The evaluation of

this possibility requires some knowledge of the kinetics of the release of the quaternary ions from the cells.

Fig. 3 shows the effect of external concentration of choline and tetramethylammonium ions on the rate of uptake by the cells. Each point represents the slope of a line obtained from an experiment similar to that shown in Fig. 2. To obtain each line, three incubation periods, none exceeding 4 hours, were chosen, and for each incubation period counts were determined on triplicate samples. For choline the results of two experiments performed with the blood obtained from two different donors are presented. Variations of this order of magnitude were obtained in two more experiments with choline and one more

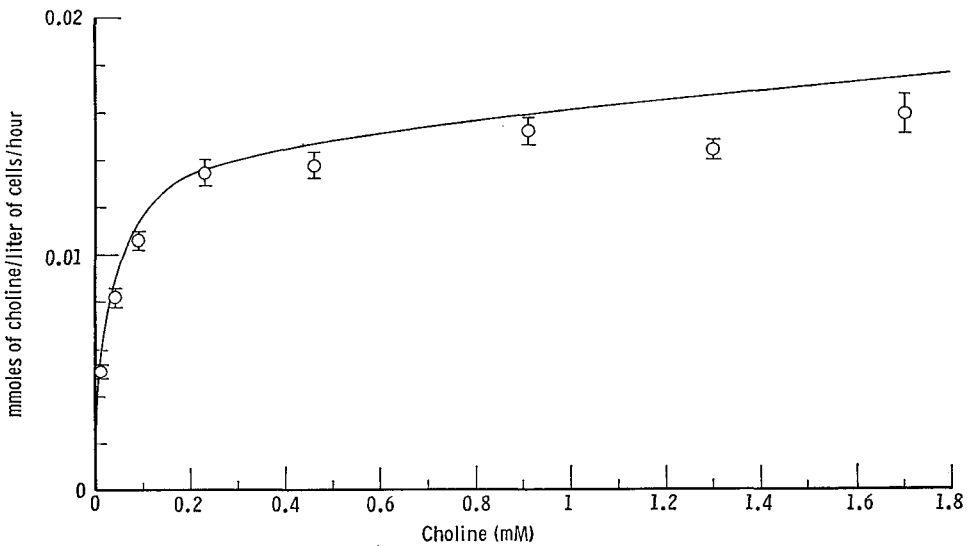

FIGURE 4. Effect of varying external concentration of choline on the rate of uptake of choline by the erythrocytes of donor A (see Fig. 3). Each vertical bar represents the range of 8 determinations. The points represent experimental values, and the line represents the equation given in the legend to Fig. 3.

with tetramethylammonium using different blood samples. Because of these differences, the data obtained with different bloods could not be combined or compared. It is evident that a straight line could be drawn through the points of Fig. 3 for each compound, showing that the increase in the rates of uptake of choline and tetramethylammonium ions is proportional to the external concentration of each compound within the range of concentrations studied. However, it is also evident that if such straight lines were drawn they would not pass through the origin, but would intercept the ordinate, indicating the presence of a different relationship between the external concentration and the rate of uptake at low concentrations. Accordingly, Fig. 4 shows the effect of considerably lower concentrations of choline on the rate of uptake. (Because of

the limited supply of C^{14}-tetramethylammonium, similar experiments were not performed with this compound.) It should be noted that the data presented in Fig. 4 were obtained with the same blood sample used in one of the experiments in Fig. 3. These data show that in this concentration range the rate of uptake of choline increases rapidly at first with increase in the external concentration of choline, but levels off as the external concentration reaches about 0.2 mM. Considering the results from both Fig. 3 and Fig. 4, it is apparent that the curve never becomes horizontal.

The results presented in Fig. 3 and Fig. 4 suggest that two distinct mechanisms are involved in the uptake of choline: one, a facilitated uptake mechanism which becomes saturated at very low concentrations of choline; the other, a simple diffusion mechanism in which the rate of uptake is proportional to concentration. Although experiments at low concentrations of tetramethylammonium, comparable to those with choline, were not performed, the fact that the straight line of Fig. 3 representing the uptake of tetramethylammonium intercepts the ordinate suggests that a facilitated uptake mechanism for this compound also exists. It is noteworthy that the results obtained with choline are qualitatively similar to those obtained by Shaw (22) and Glynn (10) in their studies on the uptake of potassium ion by horse erythrocytes and human erythrocytes. Shaw demonstrated that the curve of the rate of uptake of K^+ against the external concentration of K^+ in horse erythrocytes had a hyperbolic part and a linear part and could be fitted to an equation of the form

$$v = \frac{VS}{K_m + S} + kS,$$

where v is the rate of uptake, S the external concentration, and V, K_m, and k are constants. Without the term kS, the equation is identical with that of an an enzyme-catalyzed reaction or an adsorption isotherm, and represents the hyperbolic part of the uptake curve. The constants V and K_m have the same meanings as in enzyme kinetics, namely, the maximum rate and the concentration at which half-maximum rate is obtained. When the data on the uptake of choline were fitted in the above equation, according to the procedure of Shaw, the following approximate values for the constants were obtained: V = 0.014 mmoles per liter of cells per hour, K_m = 0.02 mM, and k = 0.003 $hour^{-1}$. The solid lines in Fig. 3 and Fig. 4 represent the equation

$$v = \frac{0.014(\text{choline}^+)}{0.02 + (\text{choline}^+)} + 0.003\ (\text{choline}^+).$$

Glynn (10), following the procedure of Shaw, obtained the following equation for the uptake of K^+ by human erythrocytes:

$$v = \frac{2.12(K^+)}{2.2 + (K^+)} + 0.006\ (K^+).$$

From these data some comparisons of possible significance can be made between the rate of uptake of choline and that of potassium in human erythrocytes. It is evident that the rates of passive diffusion of potassium and choline ions are of the same order of magnitude ($k_{pot.} = 0.006$, $k_{chol.} = 0.003$). This is not unexpected since choline is a monovalent cation with a charge density of the same order of magnitude as those of the monovalent metal ions. The great difference between choline and potassium is in the catalyzed portions of their uptake. One possible interpretation of the different values of V and K_m

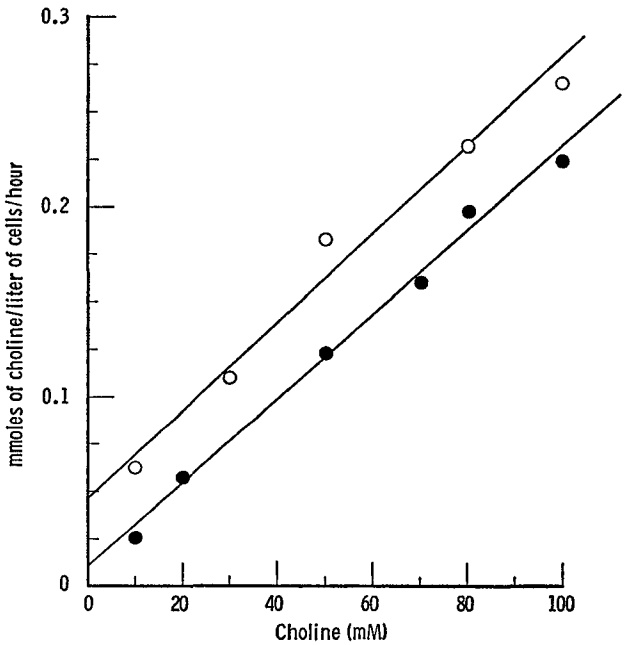

FIGURE 5. Effect of varying external concentration of choline on the rate of choline uptake in the absence (open circles) and the presence (solid circles) of 5 mM tetramethylammonium.

for the two ions is that there are a greater number of adsorption sites for K^+ than there are for choline ($V_{pot.} = 2.12$, $V_{chol.} = 0.014$); but the affinity of choline for its binding sites is about one hundred times greater than that of K^+ for its binding sites ($K_{pot.} = 2.2$, $K_{chol.} = 0.02$). Regardless of the meaning of the constants, it is evident that in a comparison of the rate of uptake of choline with those of the monovalent metal ions the relative external concentrations of the ions have to be considered. For example, from the above equations it may be calculated that at 2 mM external concentration the rate of uptake of K^+ is about a hundred times greater than that of choline; whereas at 0.02 mM the K^+ rate of uptake becomes only twice as great as that of choline.

Inhibition of Choline Uptake by Other Quaternary Ammonium Ions

Fig. 5 shows the effect of a fixed concentration of tetramethylammonium ion on the rate of uptake of choline as a function of varying external choline concentration. The approximate equality of the slopes of the two lines suggests that the only inhibitory effect of tetramethylammonium is on that portion of choline uptake which is not a linear function of external choline concentration.

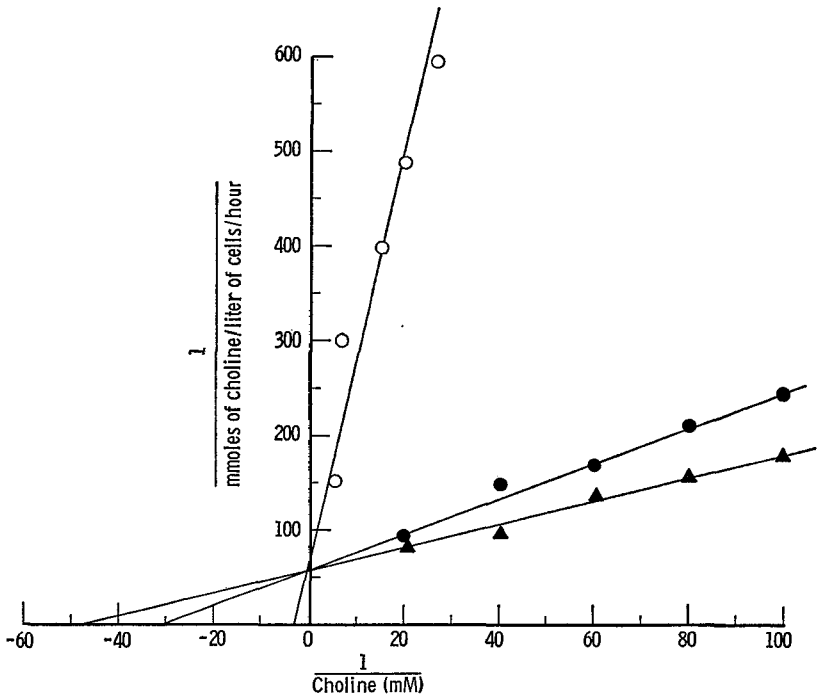

FIGURE 6. Competitive inhibition of choline uptake by tetramethylammonium and tetraethylammonium ions. The reciprocals of the rates of choline uptake in the absence of inhibitors (solid triangles), in the presence of 2 mM tetraethylammonium (solid circles), and in the presence of 2 mM tetramethylammonium (open circles) are plotted against the reciprocal of external choline concentration.

Therefore, the effects of tetramethylammonium and tetraethylammonium ions on the rate of choline uptake at low concentrations of choline were studied. The results are shown in Fig. 6, in which the reciprocal of uptake rate is plotted against the reciprocal of choline concentration. The data suggest that both tetramethylammonium and tetraethylammonium ions are competitive inhibitors of the facilitated choline uptake. Since a facilitated uptake of tetramethylammonium has already been indicated (Fig. 3), it is reasonable to conclude that the inhibition of choline uptake by other quaternary ammonium

ions is due to the fact that all these ions are taken up by the erythrocytes through the same mechanism. The inhibitor constants for tetramethylammonium and tetraethylammonium ions may be obtained from Fig. 6 by the usual methods. The calculated values are: K_{TMA} = 0.13 mM, and K_{TEA} = 3.3 mM. If these represent the dissociation constants of the ions for the facilitated uptake sites, the order of affinity of the three quaternary ammonium ions for the sites would be as follows: Choline > tetramethylammonium > tetraethylammonium.

Effects of Other Ions on the Uptake of Choline

It was of interest to know whether variations in the ionic composition of the medium had any effect on the rate of uptake of choline. Omission of either Ca^{2+}, Mg^{2+}, K^+, or all three ions from the incubation medium had no effect on the rate of choline uptake. Also, the replacement of up to 70% of the NaCl by an equiosmolar amount of sucrose did not significantly affect the rate of uptake. When more than 70% of the NaCl was replaced, owing to varying and inconsistent amounts of hemolysis, quantitative comparisons of rates of uptake were not possible.

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