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THE EFFECTS OF Al^{3+}, Cd^{2+} AND Mn^{2+} ON HUMAN ERYTHROCYTE CHOLINE TRANSPORT

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Abstract—The effects of Al^{3+}, Cd^{2+} and Mn^{2+} on human erythrocyte choline transport, Na–K-ATPase, Ca–Mg-ATPase and intracellular K^+ levels were examined. The concentrations used were below the levels which caused significant haemolysis ($\leq 300\ \mu M$). All three cations inhibited concentrative choline accumulation over 3 hr [IC_{50} values at 1 μM choline were 35 μM ($AlCl_3$), 250 μM ($CdCl_2$) and 300 μM ($MnCl_2$)] but at the concentrations tested, none decreased initial rates of choline uptake. The effects of Cd^{2+} and Mn^{2+} (but not Al^{3+}) on choline accumulation were reversed by removing the cations from the extracellular medium by washing. All three cations also inhibited efflux of choline, at 1 μM choline, 30% inhibition being produced by 33 μM $AlCl_3$, 81 μM $CdCl_2$ and 111 μM $MnCl_2$. At subhaemolytic concentrations, only $CdCl_2$ inhibited Na–K-ATPase, (IC_{50} = 147 μM) and none of the cations significantly inhibited Ca–Mg-ATPase. Intracellular K^+ levels were only reduced by the highest concentration of $AlCl_3$ used (100 μM). These results suggest that inhibition of choline accumulation and efflux in erythrocytes by Al^{3+}, Cd^{2+} and Mn^{2+} is not explicable solely in terms of either inhibition of Ca–Mg-ATPase, or inhibition of Na–K-ATPase causing reduced intracellular K^+. Our conclusions are similar to those previously obtained using synaptosomes and provide support for the hypothesis that inhibition of choline transport by Al^{3+} may contribute to a number of disease states.

Al^{3+}, Cd^{2+} and Mn^{2+} have been shown to inhibit choline uptake in synaptosomes [1] and choline transport across cell membranes is essential for the synthesis of the neurotransmitter acetylcholine [2]. All three of the above cations are neurotoxic [3, 4] and Al^{3+} and Mn^{2+} have recently been shown to be associated with adverse changes in parameters of cholinergic neuronal function. For example, chronic manganese administration to rats has been found to produce changes in levels of acetylcholinesterase and choline acetyl transferase [5]. Also raised aluminium levels have been found post mortem in the characteristic neurofibrillary tangle bearing neurones of patients with Alzheimer type senile dementia [6], a disease in which cholinergic neurones are thought to be specifically affected [7]. Therefore it is possible that Al^{3+} contributes to the pathogenesis of this disease by specifically inhibiting choline uptake by cholinergic neurones. However all three cations are also known to inhibit sodium–potassium-activated adenosine triphosphatase (ATP phosphohydrolase, E.C. 3.6.1.3 Na–K-ATPase) and calcium–magnesium-activated adenosine triphosphatase (ATP phosphohydrolase, E.C. 3.6.1.3 Ca–Mg-ATPase) [1], and might therefore affect choline transport by an indirect action on the electrochemical gradient across the cell membrane.

In order to investigate the latter possibility it was decided to study the actions of these cations on choline transport, Na–K- and Ca–Mg-ATPase activities and intracellular K^+ levels in erythrocytes. Erythrocytes possess a choline transport system which demonstrates similar properties to the choline carrier found in other cells; i.e. saturability, hemicholinium sensititivity and activation by trans choline (the transport of choline across the red cell membrane is stimulated by choline on the opposite side of the membrane) [8]. However, unlike most other cells erythrocytes do not metabolise choline; they do not synthesise acetylcholine nor do they incorporate choline into phospholipids [9]. Therefore they provide a relatively simple experiment model on which the effects of cations on efflux, influx and steady state choline levels may be investigated.

METHODS AND MATERIALS

Materials. Choline chloride and trichloracetic acid were obtained from BDH Chemicals Ltd.; Adenosine 5′triphosphate (disodium salt), Fiske and Subbarow reducer, hemicholinium-3 and ouabain octahydrate from Sigma: chloramphenicol from Parke-Davis; and [methyl-^{3}H]choline chloride (60 Ci/mmole) from Radiochemical Centre, Amersham. [Methyl-^{3}H]choline chloride was stored at −20° in ethanol, and prior to use the ethanol was removed by passing a stream of N_2 over it. All other chemicals were from Ajax Chemical Co., Sydney, and were analytical grade.

Preparation of erythrocytes. Fresh human blood was obtained from healthy volunteers (results shown are means of separate experiments with blood from different donors). Twenty milliliters blood was withdrawn into a syringe, mixed with 5 ml acid citrate dextrose medium (21 mM citric acid, 45 mM trisodium citrate, 82 mM glucose) and then centrifuged for 10 min at 1400 *g* (3000 rpm, Clements GS200 centrifuge). The plasma and buffy coat were

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removed, the red cells resuspended by gentle mixing and washed 4 times in 3 vol of "solution A" containing 147 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2.2 mM $CaCl_2$, 10 mM Tris–HCl (pH 7.4 at 37°), 5.5 mM glucose and 0.02% (w/v) chloramphenicol. Whenever a measured volume of "packed cells" was transferred to the incubation medium, the cells were first centrifuged for 20 min at 1400 *g*. The cells were used within 3 hr.

Influx experiments. The method was similar to that of Martin [8]. Cells were incubated overnight in a shaking water bath at 37° in "solution B" (as solution A, but containing 1 μM unlabelled choline chloride) to achieve "steady-state" distribution of choline. The haematocrit was 5%. They were then centrifuged as above and 0.8 ml of packed cells resuspended in 18 ml of "solution B" with or without the addition of $AlCl_3$, $CdCl_2$ or $MnCl_2$. After 1 hr 10 μCi[^{3}H] choline chloride in 2 ml of the same medium (total choline concentration 1 μM) was added and mixed. At various time intervals after the addition of [^{3}H] choline, 1 ml samples were removed and placed in centrifuge tubes on ice. The samples were immediately spun for 3 min at 2000 *g* (5000 rpm, Beckman microfuge) at 4° and the initial supernatant removed and later checked for haemolysis. The cells were resuspended by gentle vortex mixing and washed 3 times with 1 ml ice-cold "wash" buffer (as "solution A" but containing 1 mM unlabelled choline). After the final supernatant was removed 1 ml of 5% trichloroacetic acid was added, vortexed and left to stand for 1 hr. The precipitate was removed by centrifugation for 10 min at 3000 *g* and 0.4 ml of the supernatant added to 5 ml of Triton toluene scintillation medium [8 g PPO, 100 mg POPOP in 3 l. toluene: Triton 2:1 (v/v)]. The radioactivity was counted for 10 min in a Packard Prias scintillation counter.

Haemolysis was determined by measuring absorbance of the supernatant at 539 nm and comparing it with standards prepared from lysed cells.

Potassium levels. The procedure was the same as for the influx experiments except that [^{3}H] choline was replaced by unlabelled choline and after incubation the washing medium contained NaCl in place of KCl. Potassium levels in the trichloroacetic acid supernatant were measured by flame spectrophotometry.

Na–K-*ATPase and* Ca–Mg-*ATPase activity.* The method was essentially that of Bonting and Caravaggio [10] as modified by Lai *et al.* [1]. "Packed cells" prepared as above were haemolysed in 5 vol of Tris–HCl buffer (1 mM) at pH 7.5 for 15 min at 4°. Cell membranes were then centrifuged for 10 min at 5000 *g* and washed 3 times with 5 vol of 17 mM NaCl in 1 mM Tris–HCl buffer. The final sediment was resuspended in distilled water to the original volume of packed cells. Fifty microliters of this suspension were then added to assay tubes A–F with or without $AlCl_3$, $CdCl_2$ or $MnCl_2$. The final volume in each tube was 1 ml, and the final concentrations of reagents were as follows: 1 mM ATP (in A,B,C,D,E), 2 mM $MgCl_2$ (in all tubes), 5 mM KCl (in A,C,D,F), 60 mM NaCl and 92 mM Tris–HCl, pH 7.4 (in A,B,D,E,F), 15 mM Tris–HCl, pH 7.4 (in C) and 0.1 mM ouabain (in D,E). The tubes were incubated for 1 hr at 37° in a shaking water bath. Two tubes containing medium A were set up in each group of tubes. After the addition of ATP, the tubes were incubated for a further 10 min, the reaction being stopped by adding 2 ml of ice cold 10% (w/v) trichloroacetic acid. The tubes were then centrifuged at 3000 *g* for 10 min at 4° and 1 ml samples of supernatant removed for phosphate determination. To each, 1 ml of 5% (w/v) ammonium molybdate was added followed by 0.25 ml of 15% (w/v) Fiske and Subbarrow reducing agent. They were then incubated at room temperature for 10 min, and their absorbance measured at 660 nm, and compared with a series of standards prepared using KH_2PO_4.

The average activity of tubes B, C, D, and E was a measure of Ca–Mg-ATPase activity. Na–K-ATPase activity was calculated as the total ATPase activity (tubes A) minus Ca–Mg-ATPase activity. Tube F acted as a blank.

Efflux experiments. The methods and calculations were similar to those of Martin [8]. Cells were incubated overnight as for influx experiments, except that the haematocrit was 10% and the medium contained [^{3}H] choline (1 μCi/ml) and unlabelled choline (final choline concentration 1–30 μM). To remove extracellular choline the cells were centrifuged at 2000 *g* at 4° for 5 min, the supernatant discarded and the cells washed 3 times with 5 vol of ice-cold buffer (the same buffer as for overnight incubation, but unlabelled choline replacing [^{3}H] choline). Immediately after the third wash, 0.2 ml of "packed cells" were resuspended in 20 ml of the same buffer at 37° with or without the appropriate concentration of $AlCl_3$, $CdCl_2$ or $MnCl_2$, and shaken in a water bath at 37°. At various time intervals 1 ml of the cell suspension was withdrawn, placed in an ice-cold centrifuge tube, and centrifuged for 2 min at 3000 *g* (6000 rpm, Beckman Microfuge). A quantity of 0.4 ml of the supernatant was then taken for counting, and a correction was made for quenching due to any slight haemolysis present, by constructing a standard curve of efficiency of counting against % haemolysis. The radioactivity in the cells at time zero was determined by taking a 1 ml sample of cell suspension, precipitating the protein with 2 ml 10% (w/v) trichloroacetic acid, counting the supernatant, and subtracting from this the radioactivity present in the extracellular fluid at zero time. The rate constant for efflux was estimated from the slope of the graph of the logarithm of the fraction of radioactivity remaining in the cells against time (0–60 min) [8]. The initial rate of efflux was then calculated by multiplying the rate constant by the initial intracellular choline concentration [8].

Statistical analysis. This was performed by analysis of variance and Dunnett's test for multiple comparisons with a control. Linear regression and comparison of lines was performed as outlined in Documenta Geigy [11]. Concentrations of cations which caused 50 or 30% inhibition of uptake or efflux were calculated by regression over the linear portion of a graph of % activity against logarithm of cation concentration.

RESULTS

Haemolysis of erythrocytes by high concentrations of $AlCl_3$, $CdCl_2$ *and* $MnCl_2$. Initial experiments

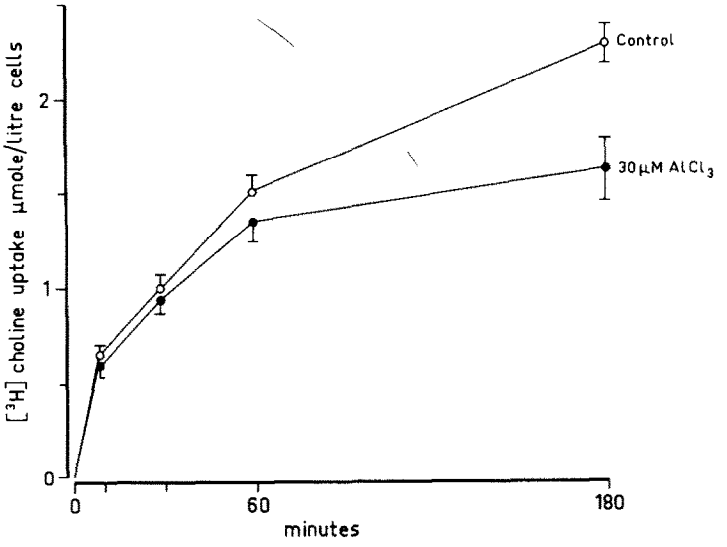

Fig. 1. The effect of $AlCl_3$ on [^{3}H] choline uptake. Uptake of [^{3}H] choline was measured as in the methods. Vertical bars show S.E. (N = 6).

showed that 1 mM $AlCl_3$, $CdCl_2$ and $MnCl_2$ all caused haemolysis of erythrocytes which was greater than 5% after 3 hr incubation at 37°. Even at concentrations of 300 μM, all three cations caused a time dependent haemolysis which was greater than 5% after 8 hr. In the case of $AlCl_3$, agglutination of red blood cells was observed at a concentration of 300 μM, but not at 100 μM. Therefore, in order to minimise the effects of haemolysis and agglutination, all subsequent experiments were performed with maximum concentrations of 300 μM $CdCl_2$, 300 μM $MnCl_2$ and 100 μM $AlCl_3$, and for periods of incubation with the cations of no greater than 6 hr.

In all these experiments the degree of haemolysis was measured, and was never greater than 5% at any time. This degree of haemolysis does not significantly affect choline transport in red cells, and is observed even after erythrocytes are incubated for 20 hr or more at 37° in buffers containing no haemolysing cations [8].

Influx of [^{3}H] *choline into erythrocytes.* Erythrocytes from different donors may have different intracellular choline levels [12]. Also the higher the intracellular choline concentration, the greater the initial rate of choline uptake into erythrocytes [8]. Therefore before influx experiments were performed, cells were washed four times in choline free buffer, and then pre-incubated for 16 hr in 100 vol of buffer containing 1 μM unlabelled choline. After this time the choline content of the cells reached equilibrium or steady state [8] and uptake of [^{3}H] choline was then measured under these same conditions (i.e. 1 μM external choline concentration).

Figure 1 shows the effect of 30 μM $AlCl_3$ on the time course of uptake of [^{3}H] choline by erythrocytes. It is seen that [^{3}H] choline uptake in the first 10 min was not significantly altered by 30 μM $AlCl_3$ whereas the total [^{3}H] choline present in the cells at 3 hr was significantly reduced ($P < 0.05$). Similar results were obtained with $CdCl_2$ and $MnCl_2$, and are summarised in Table 1, columns 1 and 2.

Erythrocytes are able to accumulate choline against an electrochemical gradient, although the mechanism of this concentrative accumulation is not known [8]. Since the packed cell volume is approximately 1.4 times the intracellular volume [13] taking into account the membrane potential of the red cell, it is argued that choline should be at thermodynamic equilibrium when the ratio concentration per volume of packed cells/extracellular concentration is 1 [8]. Results from Table 1 indicate that as the extracellular choline concentration was 1 μM there was a 2.33-fold concentration of choline by the erythrocytes after 3 hr and that Al^{3+}, Cd^{2+} and Mn^{2+} all inhibited this concentrative accumulation. A 50% inhibition of 3 hr concentrative accumulation would have therefore resulted in $1 + (2.33 - 1)/(2) = 1.665$-fold concentration of choline. From this figure IC_{50} values for the inhibition of 3 hr choline accumulation (extracellular concentration 1 μM) were calculated, and are 35 (14, 91) μM for $AlCl_3$, 250 (196, 320) μM for $CdCl_2$ and 300 (166, 550) μM for $MnCl_2$ (the figures in brackets are 95% confidence limits, $df = 16$). Thus the rank order of inhibition was $Al^{3+} > Cd^{2+} \geq Mn^{2+}$.

The period of preincubation with the cations prior to the addition of [^{3}H] choline was not critical: figures obtained for 3 hr choline uptake were not significantly different from those shown in Table 1 (column 2) for periods of preincubation of either 0, 1 or 3 hr.

An estimate of the initial [^{3}H] choline influx may be obtained by multiplying the slope of the graph of $\log [1 - (C_t)/C_e)]$ against time by C_e, where C_t and C_e are intracellular [^{3}H] choline concentrations at time t and at equilibrium respectively [8]. Because the cations caused a time dependent haemolysis as shown above, an accurate estimate of C_e was not possible at all cation concentrations. However, an estimate of the initial [^{3}H] choline influx is given by [^{3}H] choline uptake in the first 10 min. As shown in Table 1, this was not significantly affected by any of the concentrations of cations used.

Table 1. Effect of cations on [^{3}H] choline uptake

	[^{3}H] choline uptake, μmoles/l cells in			
	10 min	3 hr	3 hr (washed and resuspended in buffer as shown)	3 hr (washed and resuspended in buffer without cation)
Control	0.65 ± 0.04	2.33 ± 0.02	2.53 ± 0.10	2.47 ± 0.05
$AlCl_3$ 10 μM	0.60 ± 0.06	1.78 ± 0.21	2.02 ± 0.13*	2.23 ± 0.07*
30 μM	0.62 ± 0.05	1.65 ± 0.14*	1.64 ± 0.15*	1.85 ± 0.12*
100 μM	0.59 ± 0.03	1.60 ± 0.06*	1.52 ± 0.05*	1.65 ± 0.07*
$CdCl_2$ 30 μM	0.64 ± 0.04	2.23 ± 0.03	1.84 ± 0.15*	2.34 ± 0.12
100 μM	0.72 ± 0.03	1.81 ± 0.02*	1.89 ± 0.21*	2.30 ± 0.07
300 μM	0.67 ± 0.03	1.67 ± 0.03*	1.70 ± 0.13*	2.20 ± 0.10
$MnCl_2$ 30 μM	0.65 ± 0.01	2.02 ± 0.03	2.07 ± 0.10*	2.35 ± 0.12
100 μM	0.66 ± 0.08	1.95 ± 0.07*	1.77 ± 0.10*	2.45 ± 0.20
300 μM	0.58 ± 0.03	1.66 ± 0.07*	1.54 ± 0.05*	2.27 ± 0.07

Uptake of [^{3}H] choline (extracellular concentration 1 μM) was determined as in the methods. Results shown in columns 3 and 4 are from experiments described in the text in the section on reversibility of the effects of $CdCl_2$ and $MnCl_2$. Results show mean ± S.E. (columns 1 and 2, N = 7 except for control, N = 21: columns 3 and 4, N = 6 except for control, N = 18).

* Significantly different from control, $P < 0.05$.

Reversibility of the effects of $CdCl_2$ *and* $MnCl_2$ *on* [^{3}H] *choline uptake.* In order to ascertain whether the effects of the above cations on choline uptake were reversible, erythrocytes were incubated with the cations for 3 hr and then divided into two batches. One batch was washed four times in buffer without the cation, and the other in buffer containing the cation. Uptake of [^{3}H] choline was then measured in the same buffers as used for washing. As shown in Table 1, columns 3 and 4, cells which had been incubated for 3 hr with either 30–300 μM $CdCl_2$ or $MnCl_2$, and then washed, demonstrated a 3 hr [^{3}H] choline uptake which was not significantly different from control. Therefore the effects of these two cations on 3 hr [^{3}H] choline uptake appears to be reversible. However, in the case of $AlCl_3$, removal of extracellular $AlCl_3$ by washing did not restore the 3 hr [^{3}H] choline uptake to control values.

Na–K-*ATPase, Ca–Mg-ATPase and intracellular* K^+. The results of the above experiments on choline influx could be explained by the hypothesis that the cations are not directly inhibiting the choline transport system, but are reducing the membrane potential of the cell, possibly via inhibition of Na–K-ATPase. Choline is a positively charged ion, and therefore a reduction in membrane potential (i.e. less negative inside) would be expected to reduce intracellular choline levels at equilibrium. This could therefore explain why [^{3}H] choline uptake in the presence of the cations was found to be reduced at 3 hr (i.e. when approaching equilibrium), but not at 10 min. High concentrations of Al^{3+}, Cd^{2+} and Mn^{2+} are known to inhibit both Na–K-ATPase and Ca–Mg-ATPase in synaptosomes [1]. Measurements of these enzymes in erythrocytes were made in the presence of 10, 30, 100 μM $AlCl_3$ and 10, 30, 100, 300 μM $CdCl_2$ and $MnCl_2$.

At these concentrations, only $CdCl_2$ was found to significantly inhibit Na–K-ATPase, with an IC_{50} of 147 (77, 280) μM (mean and 95% confidence limits, $df = 10$). This figure is similar to that found by Nechay and Saunders [14], who found the IC_{50} to be 140 μM. At the cation concentrations used, we were unable to demonstrate a significant inhibition of Na–K-ATPase by $AlCl_3$ or $MnCl_2$, or an inhibition of Ca–Mg-ATPase by any of the cations. These results therefore suggested that the inhibition of [^{3}H] choline uptake by Al^{3+} and Mn^{2+} might not be due to an inhibition of Na–K-ATPase, although it could still be due to an alteration in membrane potential by some other means. To test this hypothesis, intracellular K^+ levels were measured.

Since K^+ is distributed approximately according to its electrochemical potential [15], a change in membrane potential would be expected to be reflected by a change in intracellular K^+ concentration. Incubation of erythrocytes for 4 hr with concentrations of up to 300 μM $CdCl_2$ and $MnCl_2$ did not significantly alter intracellular K^+ levels, although 4 hr incubation with 100 μM $AlCl_3$ resulted in a percentage reduction in intracellular K^+ of 51 ± 4 [mean ± S.E. (N = 6), control = 102 ± 4 mmoles/l cells]. Ten and 30 μM $AlCl_3$ did not significantly reduce intracellular K^+. Therefore, as judged by intracellular K^+ levels, 4 hr incubation with 300 μM $CdCl_2$, 300 μM $MnCl_2$ and 30 μM $AlCl_3$ did not appear to affect the membrane potential of the erythrocyte. On this evidence, the hypothesis that these cations inhibit choline uptake solely by a reduction in membrane potential does not seem tenable.

Efflux of [^{3}H] *choline from erythrocytes.* Both choline efflux and influx in erythrocytes have been shown to demonstrate similar properties, i.e. those of a carrier mediated transport system [8]. Replacement of extracellular Na^+ by K^+, thereby causing a reduction in membrane potential, has been found to reduce choline influx and total choline accumulation but increase choline efflux in erythrocytes [16]. If such effects on choline fluxes and accumulation are

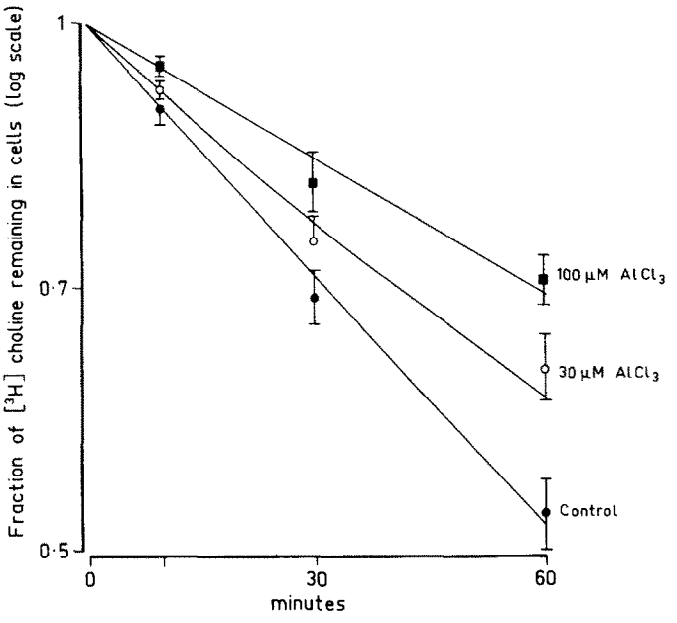

Fig. 2. The effect of $AlCl_3$ on [^{3}H] choline efflux. Efflux was measured as in the methods (extracellular choline 1 μM). Vertical bars show S.E. (N = 6).

Table 2. The effect of cations on [^{3}H] choline efflux

	Initial efflux of [^{3}H] choline nmoles/l cells/min
Control	37.2 (33.6, 40.8)
$AlCl_3$ 10 μM	38.4 (33.6, 42.9)
30 μM	26.4 (22.5, 30.3)*
100 μM	21.0 (17.4, 24.6)*
$CdCl_2$ 30 μM	29.1 (26.1, 31.8)*
100 μM	26.4 (22.8, 30.0)*
300 μM	22.2 (18.3, 25.8)*
$MnCl_2$ 30 μM	33.6 (29.4, 37.8)
100 μM	24.3 (19.8, 28.8)*
300 μM	22.2 (18.3, 25.8)*

Initial efflux of [^{3}H] choline was determined as in the methods at a steady state extracellular choline concentration of 1 μM. Figures show means and 95% confidence limits ($df = 30$) as derived from linear regression of log (fraction of radioactivity remaining in cells) against t at t = 10, 30 and 60 min.

* Significantly different from control, $P < 0.05$.

due to a depolarisation of the cell, then Al^{3+}, Cd^{2+} and Mn^{2+} would be expected to have similar effects, provided their actions are solely due to a reduction in membrane potential. Therefore it was decided to investigate the actions of these cations on [^{3}H] choline efflux from erythrocytes.

As with influx experiments, choline efflux was measured at steady state. Erythrocytes were washed four times in choline free buffer and loaded with radiolabelled choline by preincubating them for 16 hr in buffer containly 1–30 μM [^{3}H] choline. A buffer containing the same concentration of unlabelled choline was then used to wash the cells, and also to suspend the cells for efflux determination. There was no preincubation of the cells with cations prior to efflux, as comparison of results depended on identical initial intracellular choline concentrations, which have been shown to be affected by prior incubation with the cations.

As shown in Fig. 2, 30 and 100 μM $AlCl_3$ inhibit choline efflux. Initial [^{3}H] choline efflux was calcu-

Table 3. Choline efflux at various choline concentrations

	Initial efflux of [^{3}H] choline nmoles/l cells/min at an extracellular choline concentration of			
	1 μM	3 μM	10 μM	30 μM
Control	44.7 (38.7, 50.7)	125 (109, 141)	370 (320, 420)	890 (790, 990)
100 μM $AlCl_3$	34.2* (28.2, 40.2)	93.2* (81.6, 104.7)	274* (223, 324)	722* (610, 834)
300 μM $CdCl_2$	27.3* (22.5, 32.1)	91.6* (86.2, 97.0)	386 (276, 496)	862 (761, 963)
300 μM $MnCl_2$	28.8* (22.5, 35.1)	73.9* (57.0, 90.8)	243* (186, 301)	677 (492, 862)

Initial efflux of [^{3}H] choline was determined as in the methods. Figures show means and 95% confidence limits ($df = 14$).

* Significantly different from control, $P < 0.05$.

lated as in the methods, and results obtained at an extracellular choline concentration of 1 μM are shown in Table 2. Even at the highest concentrations of cations used, the inhibition of choline efflux was never greater than 50%, therefore it was not possible to calculate IC_{50} values (i.e. concentrations of the cations which caused a 50% reduction in choline efflux). As discussed above, higher concentrations of these cations could not be used because they caused haemolysis or agglutination. However, concentrations of cations which caused 30% inhibition of choline efflux could be calculated. These values for $AlCl_3$, $CdCl_2$ and $MnCl_2$ were 33 (14, 77) μM, 81 (55, 120) μM, and 111 (84, 146) μM respectively, where the figures in brackets are 95% confidence limits ($df = 16$). Thus the rank order of inhibition was $Al^{3+} > Cd^{2+} > Mn^{2+}$.

In Table 3 are shown the results of preloading the cells with different concentrations of [^{3}H] choline under steady state conditions. The highest concentration used was 30 μM [^{3}H] choline, since it has been shown that at choline concentrations of 50 μM or more, erythrocytes do not equilibrate within 16 hr, and longer equilibration times result in substantial haemolysis [8]. Also at such high choline concentrations, the ratio of choline which crosses the membrane by simple diffusion to transported choline becomes substantial [17]. From Table 3 it is seen that 300 μM $CdCl_2$ and $MnCl_2$ significantly inhibit [^{3}H] choline efflux only at choline concentrations of 1 and 3 μM ($CdCl_2$) and 1, 3 and 10 μM ($MnCl_2$). At 30 μM choline, neither of these cations significantly inhibited efflux. However in the case of $AlCl_3$, [^{3}H] choline efflux was significantly inhibited at all concentrations of choline used.

Choline transport in erythrocytes has been found to be stimulated by trans choline i.e. the transport of choline across the red cell membrane is stimulated by choline on the opposite side of the membrane [8]. Thus under steady state conditions an increase in choline concentration on both sides of the membrane would be expected to have two opposing effects on the choline carrier: (1) to stimulate choline transport by transactivation, as discussed above, and (2) to decrease the ratio of transported choline/choline concentration, because the carrier is saturable. Therefore under steady state conditions, the choline transport system would not be expected to demonstrate Michaelis–Menten kinetics. Indeed it has been shown that Lineweaver–Burk plots of choline efflux at steady state are non-linear [8]. Thus it is not possible to analyse the data presented in Table 3 in terms of Michaelis–Menten kinetics. However, both $CdCl_2$ and $MnCl_2$ exhibit properties of competitive inhibitors of choline transport, since the percentage inhibition of choline efflux at a fixed cation concentration decreased with increasing choline concentration and is not significantly different from control at the highest choline concentration (30 μM). $AlCl_3$ does not exhibit the above properties, and therefore on this evidence cannot be said to be a competitive inhibitor of choline transport.

Effect of cations in the presence of hemicholinium-3. A non-specific decrease in membrane permeability caused by $AlCl_3$, $MnCl_2$ or $CdCl_2$ could account for the decrease in choline efflux and 3 hr uptake. If this were so, then the cations would be expected to reduce choline efflux and 3 hr uptake even in the presence of a specific choline transport inhibitor such as hemicholinium-3. Conversely, if the cations are acting only on the choline carrier, they would not produce any further inhibition of choline efflux and influx when the carrier was maximally inhibited (e.g. by 1 mM hemicholinium-3 at a choline concentration of 1 μM, [8]). Therefore effects of the cations on both efflux and influx were measured in the presence of 1 mM hemicholinium-3 (HC-3) at an extracellular choline concentration of 1 μM. The values for 3 hr uptake of [^{3}H] choline (μmoles/l of cells, mean ± S.E., N = 6) were 1.01 ± 0.05 (HC-3 alone), 0.85 ± 0.04 (HC-3 plus 100 μM $AlCl_3$), 1.16 ± 0.03 (HC-3 plus 300 μM $CdCl_2$) and 1.15 ± 0.06 (HC-3 plus 300 μM $MnCl_2$). The percentage inhibition of 3 hr concentrative accumulation of choline produced by HC-3 was 99%, but there was no significant difference between 3 hr uptake in the presence of HC-3 alone and in the presence of HC-3 plus any of the cations. The values for initial efflux of [^{3}H]choline (nmoles/l cells/min, mean and 95% confidence limits, $df = 16$) were 2.0 (1.5, 2.5) in the presence of HC-3 alone, 1.9 (1.4, 2.4) for HC-3 plus 100 μM $AlCl_3$, 2.2 (1.6, 2.8) for HC-3 plus 300 μM $CdCl_2$ and 2.0 (1.4, 2.6) for HC-3 plus 300 μM $MnCl_2$. There was no significant difference between initial efflux in the presence of HC-3 alone and in the presence of HC-3 plus any of the cations. Therefore, concentrations of cations which have been shown to produce marked inhibition of choline efflux and 3 hr uptake had no effect in the presence of 1 mM hemicholinium-3. Thus it appears that the cations do not produce their effect on choline transport by a non-specific action on membrane permeability.

DISCUSSION

Al^{3+}, Cd^{2+} and Mn^{2+} have been found to inhibit choline uptake by synaptosomes [1], but in these experiments no measure was made of the degree of agglutination or lysis of the synaptosomes. The concentrations of Al^{3+}, Cd^{2+} and Mn^{2+} found by these workers to cause 50% inhibition of choline uptake in synaptosomes would have caused agglutination or haemolysis in red blood cells, and therefore it is possible their effects on synaptosomes were not due to actions on the choline transport system *per se*, but rather to non specific lysis or agglutination. However, by restricting the concentrations of cations used to levels which did not cause significant haemolysis or agglutination in red blood cells, our results show that these cations inhibit choline transport by a more specific mechanism. The rank order of inhibition of choline efflux from red blood cells as judged by the concentrations which caused a 30% inhibition ($Al^{3+} > Cd^{2+} > Mn^{2+}$) is similar to the rank order of inhibition of concentrative choline accumulation ($Al^{3+} > Cd^{2+} \geq Mn^{2+}$), which is the same as that found in synaptosomes [1]. Also our results on the effects of these cations on Na–K-ATPase and Ca–Mg-ATPase are comparable to those found in synaptosomes, where the only cation found to cause 50% inhibition at a concentration of $\leq$300 μM was Cd^{2+}, acting on Na–K-ATPase.

From these results, the mechanism of action of Al^{3+} and Mn^{2+} on choline transport does not appear to be via an inhibition of Ca–Mg-ATPase, Na–K-ATPase or an alteration in membrane potential. However the action of Cd^{2+} might be mediated via inhibition of Na–K-ATPase, even though no intracellular K^+ changes were detected during the course of the experiments. If the cations are inhibiting choline transport by a direct action on the carrier (which seems likely in the case of Al^{3+} and Mn^{2+}), then why was efflux inhibited, whereas no effects were observed on initial rates of choline uptake? A possible explanation may be because initially the cations only have access to the extracellular surface of the red cell membrane, and in so doing may preferentially inhibit efflux. A similar observation has been made by Edwards [18] in the case of *N*-ethylmaleimide, which inhibits choline transport in red blood cells. This author concludes that *N*-ethylmaleimide reacts with the inward facing carrier, but not the outward facing carrier, i.e. there is an asymmetry of the choline carrier which is observed in its interaction with this inhibitor. Also, the action of lithium on erythrocyte choline levels is postulated to be due to a preferential inhibition of efflux as opposed to influx [12].

The concentrations of Cd^{2+} and Mn^{2+} found to cause 30% inhibition of choline efflux or accumulation are considerably higher than those found physiologically (normal human blood values of both cations $< 1\ \mu M$ [3]) and therefore their actions on choline transport would only be expected to be of clinical significance in exceptional circumstances with markedly raised plasma levels. However, in the case of Al^{3+}, we have found significant effects on choline transport at concentrations of 10–30 μM i.e. concentrations not greatly in excess of the physiologically normal (3–7 μM [19]). This raises the possibility that in disease states where elevated aluminium levels have been found (e.g. Alzheimer's senile dementia [6] and dialysis dementia [20]), aluminium may be contributing to the pathology by inhibition of choline transport. Indeed, in Alzhiemer's dementia, both raised aluminium levels and deficits in parameters of cholinergic neuronal function have been found [7].

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