Br. J. Pharmac. (1969), **36**, 458–469.

Effects of quaternary ammonium compounds on choline transport in red cells

K. MARTIN

Department of Pharmacology, University of Cambridge, Downing Street, Cambridge

1. Neuromuscular blocking agents have been shown to be powerful inhibitors of choline transport in human erythrocytes. Ganglionic blocking agents were weaker inhibitors.

2. The affinity of the choline transport site for alkyltrimethylammonium compounds was considerably higher than its affinity for alkyl-*bis*-(trimethylammonium) compounds of similar chain length. The affinity increases with increasing length of the alkyl chain in both series.

3. Tetramethylammonium (TMA), ethyl- and propyltrimethylammonium appear to enter the cells on the choline carrier while the larger monoquaternary compounds, and the bisquaternary compounds, bind to the carrier but are unable to cross the cell membrane.

4. Radioactively labelled carbachol, acetylcholine and decamethonium do not enter the cells on the choline carrier.

5. Choline transport in erythrocytes from patients with myasthenia gravis is normal, suggesting that this disorder is not associated with a generalized defect of choline transport.

Two recent papers (Askari, 1966 ; Martin, 1968) have established the existence of a choline transport system in human erythrocytes. As an experimental preparation erythrocytes offer many obvious advantages, and when preliminary experiments with inhibitors had shown that the choline transport system of these cells is in many respects similar to that found in nervous tissue (compare Hodgkin & Martin, 1965 ; Matthews, 1966 ; Marchbanks, 1968) it seemed worthwhile to study it in more detail. This paper describes the effects of various quaternary ammonium compounds on choline transport. One object of these experiments was to compare the affinity of the choline transport site for different inhibitors with that of other sites interacting with choline—for example cholinesterase and various cholinergic receptors. In addition, it was hoped that knowledge of how this transport system handles molecules that are structurally related to choline might give some information concerning the properties of the " carrier " involved. For such a study erythrocytes are particularly useful because they can be loaded with radioactively labelled choline and the efflux of this can be increased by adding to the external medium choline or another compound that can enter the cells on the choline carrier. On the other hand the efflux of labelled choline from the cells decreases when a non-penetrating inhibitor

is added to the external medium. This counterflow phenomenon makes it possible—without having to label the inhibitors—to differentiate between molecules that can cross the cell membrane on the choline carrier and those that simply bind to it. It was therefore possible to obtain some information on the structural features determining the affinity for the transport site and whether a molecule is transported or not.

The paper also describes some experiments with erythrocytes from patients with myasthenia gravis.

A preliminary report of part of this work has been made to the Physiological Society (Martin, 1969).

Methods

The procedure for preparing the cells and for measuring choline influx and efflux were as described in a previous paper (Martin, 1968). Fresh blood was used in all experiments.

The data used to calculate the affinity of an inhibitor for the choline transport system were obtained by measuring the influx of labelled choline in the steady state at various choline concentrations in the absence and presence of the inhibitor in the external medium. The cells were added to the medium containing radioactive choline and the inhibitor. The intracellular radioactivity was determined after incubation for 15, 30 and 60 min and from these values the fluxes were calculated as described previously. The procedure for measuring the uptake of radioactive tetramethylammonium, carbachol, acetylcholine and decamethonium was similar to that for determining choline influx except that the cells had not been pre-equilibrated with the respective unlabelled compound. These fluxes are therefore not fluxes in the steady state.

Chromatography. In some experiments erythrocytes were incubated with methyl-^{14}C acetylcholine and the radioactivity taken up by the cells was identified by chromatography. The washed cells were precipitated with ethanol and 20 μl. aliquots of the supernatant were applied to Whatman No. 1 filter paper, together with appropriate standards of choline and acetylcholine chloride. The chromatograms were developed by ascending chromatography for approximately 16 hr with the following solvent: *n*-butanol, glacial acetic acid, H_2O, ethanol, 80 : 10 : 30 : 20 (v/v). The R_f values were 0·31 for choline chloride and 0·41 for acetylcholine chloride (Lewin & Marcus, 1965). The dried chromatograms were sprayed with an iodoplatinate reagent (Marchbanks, 1968). Choline and acetylcholine appear as dark blue spots on a pink background. The chromatograms were then cut into strips and the radioactivity was located with a Radiochromatogram Scanner (Packard, Model 7200).

Materials. The following isotopes were obtained from the Radiochemical Centre, Amersham: 32 mc/m-mole choline chloride (methyl ^{14}C); 11 mc/m-mole acetyl-1-^{14}C choline chloride; 10·4 mc/m-mole acetyl choline (methyl ^{14}C); 8 mc/m-mole carbamoyl choline chloride (methyl ^{14}C); 7 mc/m-mole tetramethylammonium chloride (methyl ^{14}C); 12 mc/m-mole decamethonium bromide (methyl ^{14}C). The isotopes were stored at $-10°$ C and in the case of choline and acetylcholine the purity examined as previously described (Martin, 1968).

Hemicholinium (HC-3) was a gift from Professor F. C. MacIntosh, McGill University; the alkyltrimethylammonium compounds were a gift from Professor A. S. V. Burgen, University of Cambridge, the alkyl-*bis*-(trimethylammonium) compounds a gift from Dr. R. B. Barlow, University of Edinburgh. Tris-(hydroxymethyl) aminomethane base and hydrochloride were obtained from Sigma Chemical Company; diisopropylphosphofluoridate (DFP) from Boots Pure Drug Company. The other chemicals were B.D.H. reagents.

Results

Effects of some neuromuscular and ganglionic blocking agents

If a molecule with a significant affinity for the choline transport site is added to the incubation medium it will inhibit the influx of choline. The kinetics of choline influx in the steady state can be described by the Michaelis equation (Martin, 1968) and it is therefore possible to distinguish between competitive and non-competitive inhibition and to calculate the affinity constant for the inhibitor by measuring the inhibition at various concentrations of choline. The results from such experiments were analysed by plotting S/V against S (where S is the concentration of choline in the medium and V the flux of choline). The affinity constants for choline and the inhibitor were determined from the values of the appropriate intercepts and the maximum flux, V_m is given by the reciprocal of the slope of the lines fitted to the experimental points (Dixon & Webb, 1964). This method of plotting the Michaelis equation was used because it gives an accurate intercept with the abscissa even though most measurements were obtained with choline concentrations that were below the dissociation constant (K_m) (Fig. 1). The measurements were restricted to these low—though physiological—choline concentrations because of the long time taken for red blood cells to reach a steady state when placed in solutions containing high concentrations of choline (Martin, 1968). On the other hand, for reasons to be discussed later, it was desirable to study the effects of inhibitors in the steady state because this made it possible to compare the inhibition of the influx with the inhibition of the corresponding efflux.

The affinity constants for a number of physiologically interesting substances (Table 1) were obtained in this way: by measuring the inhibition of choline influx during 1 hr. These results did not indicate whether the inhibitor could enter erythrocytes on the choline carrier or whether it was simply bound to the transport site at the outside of the membrane. It appears that neuromuscular blocking agents like D-tubocurarine, decamethonium and suxamethonium are powerful inhibitors of choline transport while the ganglionic blocking agents hexamethonium and tetraethylammonium have much lower affinities. Physostigmine and atropine are also very weak inhibitors.

It would be interesting to know the affinity of the transport site for acetylcholine (ACh) and attempts were made to determine it by measuring the effect of ACh on choline uptake into red cells that had been incubated for 20 min with 10^{-5}M DFP. The affinity constant for ACh obtained in this way was virtually the same as that for choline, suggesting that the transport system is unable to differentiate between these two molecules (Martin, 1969). However, experiments with radioactive ACh revealed that in these conditions the residual cholinesterase activity is sufficient to split most of the ACh present. Since inhibition of acetylcholinesterase by organo-

phosphorus compounds is progressive, another attempt at blocking the cholinesterase activity was made by incubating erythrocytes overnight with 10^{-5}M DFP. This treatment reduces the cholinesterase activity to about 3–4% of the original value (Burgen & Hobbiger, 1951) and leads to the conversion of reactivatable into non-reactivatable phosphorylated acetylcholinesterase (Hobbiger, 1956). However, it turned out that this treatment also reduced choline transport to about 20% of the

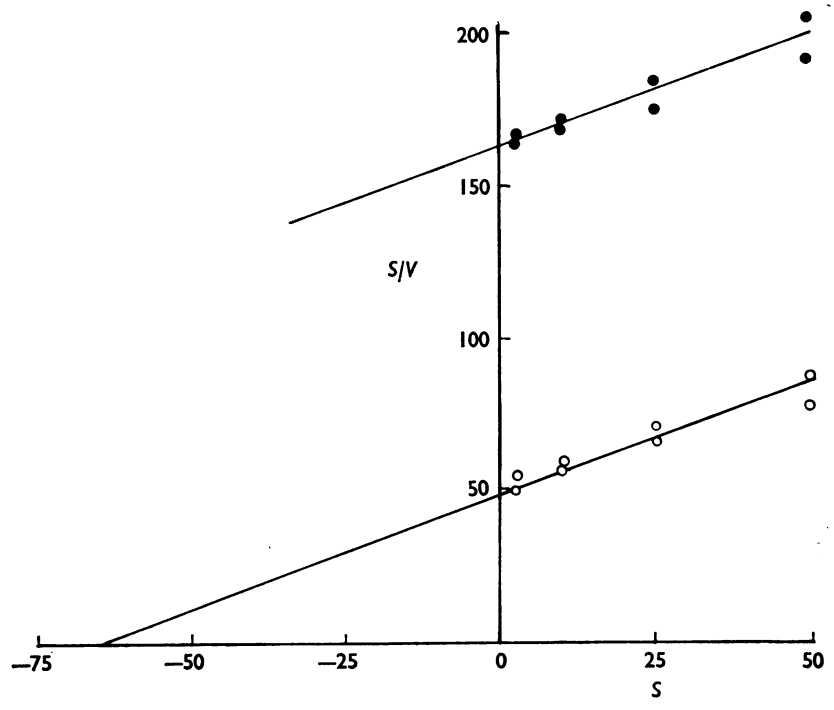

FIG. 1. Choline influx in the steady state in the absence (○) and presence (●) of decamethonium (2×10^{-4}M) in the incubation medium. S is the concentration of choline (μM) in the medium and V is the choline flux into the erythrocytes (μ-mole/l. cells per min). The data are from two experiments with blood from the same donor. The line fitted to the control (○) intercepts the abscissa at $-K_m$ ($=-62$ μM); K_m is the dissociation constant for choline. The line fitted to the solid points intercepts the abscissa at $-K_m(1+i/K_i)$ ($=-208$ μM); i is the concentration of the inhibitor and K_i its dissociation constant. The slope of the lines is $1/V_m$ where V_m is the maximum flux of choline (in this case 0·80 μ-mole/l. cells per min).

TABLE 1. *Affinity constants for the choline transport site.*

Compound	Affinity constant, K(M^{-1})
Hemicholinium HC-3	$2{\cdot}5 \times 10^5$
Choline	$2{\cdot}1 \times 10^4$
Decamethonium	$1{\cdot}2 \times 10^4$
Suxamethonium	$6{\cdot}7 \times 10^3$
D-Tubocurarine	$5{\cdot}0 \times 10^3$
Tetramethylammonium	$3{\cdot}5 \times 10^3$
Dimethyl tubocurarine	$2{\cdot}6 \times 10^3$
Carbachol	$2{\cdot}3 \times 10^3$
Physostigmine	$2{\cdot}0 \times 10^3$
Tetraethylammonium	$2{\cdot}5 \times 10^2$
Hexamethonium	$2{\cdot}1 \times 10^1$
Atropine	$<10^4$

The affinity constants are the reciprocals of the dissociation constants.

control. No further attempts were made to determine the affinity for ACh. Experiments relevant to the question whether the system can transport ACh are presented and discussed later.

In the steady state, the influx and efflux of choline are equal and an inhibitor that acts by binding to the carrier without being transported itself will, according to most transport models (for a discussion of various models see Stein, 1967), inhibit influx and efflux to the same extent. On the other hand, if the inhibitor is transported it may, when added to the external medium, either inhibit efflux in the steady state (though less than the corresponding influx) or actually increase the efflux if the system shows a pronounced counterflow phenomenon (Wilbrandt & Rosenberg, 1961; Stein, 1967). This counterflow or exchange flux phenomenon has been demonstrated for hexose transport mechanisms (see, for example, Morgan, Regen & Park, 1964) and also for the choline transport system (Martin, 1968).

When the inhibitors listed in Table 1 were examined by comparing their effect on choline influx and efflux it was found that all of them inhibited the two fluxes to the same extent except tetramethylammonium (TMA) which increased the efflux but inhibited the influx. This suggested that TMA could enter the cells on the choline transport system. Experiments in which cells were incubated with radioactive TMA confirmed that it was taken up into the cells. Figure 2 shows

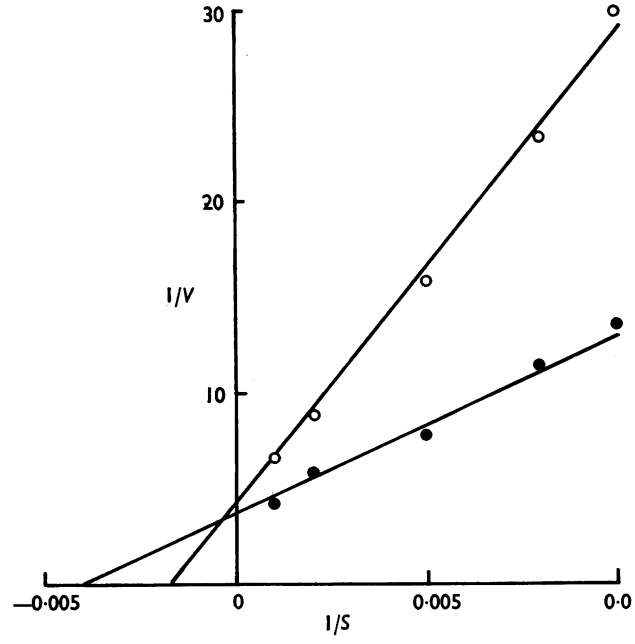

FIG. 2. Influx of TMA in the absence (●) and presence (○) of choline (6×10^{-5}M) in the incubation medium. S is the concentration of TMA (μM) in the incubation medium and V is the influx of TMA (μ-mole/l. cells per min). The line fitted to the solid points intercepts the abscissa at $-1/K_m$; K_m (250 μM) is the dissociation constant for TMA. The line fitted to the open points intercepts the abscissa at $-1/K_m\,(1+i/K_i)$; i is the concentration of choline and K_i its dissociation constant. K_i comes to 5×10^{-5}M. The intercepts of the two lines with the ordinate is $1/V_m$ where V_m is the maximum flux of TMA (in this case 0·25 μ-mole/l. cells per min). This is a maximum flux for non-steady state conditions; it is therefore not comparable with the V_m for choline based on the data in Fig. 1 (see Martin, 1968).

Lineweaver-Burk plots of experiments measuring the uptake of labelled TMA in the absence and presence of choline. The affinity constant for TMA calculated from these data is $4 \times 10^3 M^{-1}$ and agrees well with the value in Table 1, which was calculated from the inhibitory effect of TMA on choline influx. The affinity constant for choline calculated from its inhibitory effect on TMA influx is $2 \times 10^4 M^{-1}$ which again agrees with the value in Table 1 based on the influx of choline. It seems therefore that TMA enters on the choline carrier and increases choline efflux in the same way that external choline does (Martin, 1968).

Effects of alkyltrimethylammonium and alkyl-bis-trimethylammonium compounds

The fact that decamethonium (C_{10}) is a much more powerful inhibitor of choline transport than hexamethonium (C_6) raises the question whether a certain distance between the two quaternary nitrogen atoms is optimal for combination with the transport site. To study this question the affinity constants for a number of bisquaternary compounds, ranging from $(Me)_3N\text{-}(CH_2)_6\text{-}N(Me)_3$ to $(Me)_3N\text{-}(CH_2)_{18}\text{-}N(Me)_3$ were calculated from experiments measuring their inhibition of choline influx (Fig. 3). It seems that over this range the affinity increases as the carbon chain becomes longer and there is no evidence for an optimal distance between the two charged groups.

The next question is whether there is any evidence that the second quaternary nitrogen interacts either with the carrier or a neighbouring molecule in such a way as to increase the inhibitory potency. A comparison between mono- and bisquaternary compounds (Fig. 3) reveals that for the same chain length a monoquaternary compound is a more powerful inhibitor and this difference is more pronounced the shorter the chain length. The high affinities of certain bisquaternary compounds for

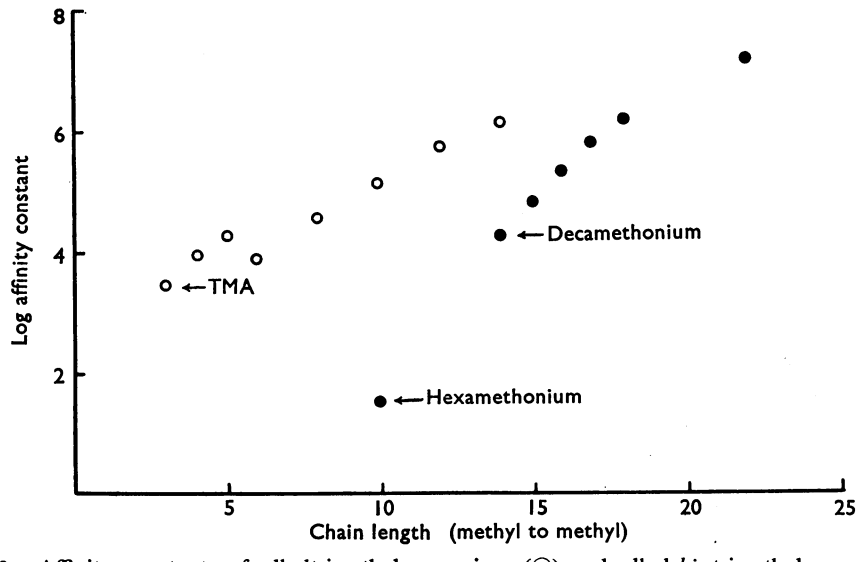

FIG. 3. Affinity constants of alkyltrimethylammonium (○) and alkyl-*bis*-trimethylammonium (●) compounds for the choline transport system. The positive $\log_{10}$ of the affinity constant is plotted against the chain length of the molecule. The chain length is expressed as the number of atoms forming the chain, including the nitrogen and the carbon of the terminal methyl group (for example TMA has a chain length of 3, hexamethonium a chain length of 10).

the neuromuscular or ganglionic receptor and the existence of an optimum distance between the two quaternary nitrogens (Paton & Zaimis, 1949, 1951) are often taken as evidence for a negative " anchoring " site on or near the receptor. The results shown in Fig. 3 give no evidence for a similar situation at the choline transport site.

If it is assumed that the relationship between the affinity constant, K, and the standard free energy change, ΔF, on adsorption of the molecule to the carrier is given by

$$\Delta F = -RT\ 2{\cdot}3\ \log_{10} K$$

one can calculate the increment in ΔF associated with the addition of a CH_2 group to the molecule. Between CH_3-$(CH_2)_3$-$N(Me)_3$ and CH_3-$(CH_2)_{14}$-$N(Me)_3$ the affinity of monoquaternary compounds increases without an apparent break in the curve. Over this range the average increment in ΔF for one CH_2 group added is -360 cal/mole. For the bisquaternary compounds the increment is larger, about -860 cal/mole averaged over the whole range tested. When a compound is partitioned between water and a non-polar solvent the free energy change associated with the addition of a CH_2 group is usually greater, between -600 and -800 cal/mole (Kauzman, 1959).

All bisquaternary compounds inhibited choline influx and efflux to the same extent; the same was true for monoquaternary compounds from butyl- to dodecyltrimethyl-ammonium. However, the three short chain alkyltrimethyl compounds inhibited choline influx—the affinity constants given in Fig. 3 have been calculated from this effect—while increasing the efflux of choline (Table 2). It has been argued in this paper and previously (Martin, 1968) that the increase in efflux of labelled choline that occurs when unlabelled choline or TMA is added to the external medium is probably a counterflow phenomenon—a phenomenon associated with the transport of these molecules into the cells. One is therefore tempted to argue similarly that ethyl- and propyltrimethyl-ammonium increase choline efflux because they can enter the cells on the choline carrier and that molecules with a larger chain fail to increase choline efflux because they are too big to enter on the carrier. Consistent with this idea are results obtained with labelled ACh and carbachol.

The calculation of the affinity constant for a penetrating inhibitor is complicated by two factors: the inhibitor will disturb the steady state for choline and it may have a different affinity for the carrier on either side of the membrane. To mimimize errors arising from this, the affinity constants for the monoquaternary com-

TABLE 2. *Efflux of choline in the absence and presence of various compounds in the external medium.*

Choline efflux (μ-mole/min per l. cells)	In external medium	
0·143	—	
0·556	$(CH_3)_3$ N-CH_2-CH_2-OH(Choline)	10^{-4}M
0·543	$(CH_3)_3$ N-CH_3 (TMA)	5×10^{-3}M
0·533	$(CH_3)_3$ N-CH_2-CH_3	10^{-3}M
0·545	$(CH_3)_3$ N-CH_2-CH_2-CH_3	10^{-3}M
0·066	$(CH_3)_3$ N-CH_2-CH_2-CH_2-CH_3	10^{-3}M
0·095	$(CH_3)_3$ N-CH_2-CH_2-O-CO-NH_2 (Carbachol)	2×10^{-3}M

The erythrocytes were incubated overnight with radioactively labelled choline and contained 48 μ-moles choline/l. cells at the beginning of the efflux experiment

pounds (Fig. 3) was determined from choline fluxes during short incubation periods (samples taken at 5 and 10 min). During this time, only small amounts of inhibitor will have entered the cells. Furthermore, in the case of TMA the affinity constant was independently evaluated by measuring the influx of radioactive TMA into cells from the same donor. The two values agreed well, suggesting that the affinity constant for a penetrating inhibitor can be satisfactorily determined from the inhibition of choline influx during short incubation periods.

Experiments with labelled ACh and carbachol

Studies with labelled ACh are complicated by the powerful cholinesterase of human erythrocytes. As pointed out earlier it was not possible to inhibit the cholinesterase completely without at the same time interfering with the choline transport system. With physostigmine sulphate ($1{\cdot}5 \times 10^{-4}$M) the uptake of choline (10^{-5}M in the external medium) was reduced by 20%. On the other hand, when cells were added to a medium containing physostigmine ($1{\cdot}5 \times 10^{-4}$M) and ACh (2×10^{-5}M) there was still evidence of acetylcholinesterase activity, because chromatographic analysis of the incubation medium 10 min after addition of the cells showed that the radioactivity was no longer associated with ACh. During these 10 min some radioactivity was taken up into the cells but there was no evidence that it entered as ACh. Indeed, when the ACh in the incubation medium was acetyl-labelled, the uptake of radioactivity was not inhibited by high concentrations of choline or hemicholinium (Fig. 4). Further, the radioactivity recovered from cells incubated for 10 min with choline-labelled ACH turned out to be associated with choline when examined by chromatography.

Carbachol is a poor substrate for acetylcholinesterase. When red cells were incubated with radioactive carbachol, there was a slow uptake of radioactivity that was insensitive to both choline and hemicholinium (Fig. 4), suggesting that carbachol entry did not depend on the choline transport system. Consistent with this is the observation that unlabelled carbachol in the external medium reduced slightly the efflux of labelled choline (Table 2). A transport system in rat brain slices capable of accumulating carbachol has been described by Creese & Taylor (1967).

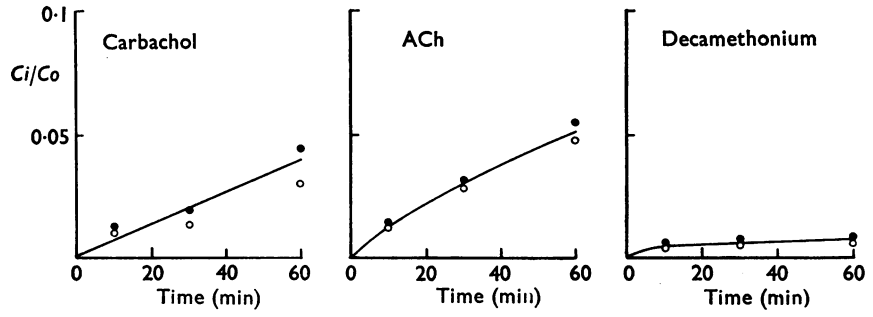

FIG. 4. Uptake of radioactive carbachol, acetate-labelled ACh and decamethonium. C_i is the radioactivity per volume of erythrocytes, C_o the radioactivity per volume of external medium. The uptake was measured in the absence (●) and presence (○) of 10^{-3}M hemicholinium (HC-3). The concentration of the labelled compound was in all three cases between 2 and 3 μM.

Creese & Maclagan (1967) reported that decamethonium can enter muscle cells by a saturatable mechanism. When erythrocytes were incubated with radioactive decamethonium there was only a very slow, hemicholinium-insensitive uptake of radioactivity (Fig. 4). These results, together with the observation that decamethonium inhibits choline influx and efflux to the same extent indicate that the choline transport system described here is not capable of transporting decamethonium.

Choline uptake by erythrocytes from patients with myasthenia gravis

The muscular weakness in myasthenia gravis is primarily the result of a failure of neuromuscular transmission and there is evidence suggesting that this is due to a deficiency in the amount of ACh released from the terminal of the motor nerve (Dahlbäck, Elmqvist, Johns, Radner & Thesleff, 1961; Desmedt 1958, 1966; Elmqvist, Hofman, Kugelberg & Quastel, 1964). Desmedt (1966) suggested as a model for the myasthenic lesion a defect in ACh synthesis such as may result from a disorder of choline transport. Since the choline transport in human erythrocytes is in many respects similar to that found in excitable tissue (Birks & MacIntosh, 1961; Hodgkin & Martin, 1965; Matthews, 1966) it seemed worthwhile to study the choline uptake in erythrocytes from patients with myasthenia gravis to see whether there is any indication of a generalized defect of choline transport.

Blood was obtained from six patients with myasthenia gravis. Three of these were moderate cases and three were severe cases, two of them with respiratory failure. Two patients were studied before and 1 week after their thymus was removed. The washed erythrocytes were incubated with 10 μM-labelled choline and the rate of influx calculated from samples taken at 10, 30, 60 and 120 min. To see whether the cells were able to concentrate choline the intracellular radioactivity was also measured when it had become constant 16 to 20 hr after the start of the incubation. Control blood was obtained from four patients of similar age and general health but not suffering from any neuromuscular disease. The initial rate of choline influx was $0{\cdot}081 \pm 0{\cdot}03$ μ-mole/l. cells per min for the cells obtained from patients with myasthenia, $0{\cdot}085 \pm 0{\cdot}025$ μ-mole/l. cells per min for the control group. The measurements at 16 and 20 hr gave no indication that the ability to concentrate choline was impaired: the ratio of cellular radioactivity over radioactivity in the external medium was $1{\cdot}46 \pm 0{\cdot}21$ for patients with myasthenia, $1{\cdot}35 \pm 0{\cdot}28$ for the control group.

To see whether dialysed plasma from myasthenics inhibited choline transport, the uptake of radioactive choline by erythrocytes incubated in their own plasma was measured after the plasma was equilibrated with a buffer containing 10 μM choline; samples were taken at 1 hr, 2 hr, 16 hr and 20 hr. The results indicated that erythrocytes from myasthenics and from controls took up choline at the same rate and concentrated it to the same extent, whether they were incubated in their own dialysed plasma or washed and incubated in an isotonic salt solution. It therefore seems that at physiological concentrations of choline the uptake of radioactive choline by erythrocytes from myasthenics is indistinguishable from the controls. This, of course, does not necessarily mean that choline uptake into nervous tissue is also unaffected in myasthenia but argues against a generalized defect in choline transport. A normal choline uptake in myasthenia is consistent with the observa-

tions made by Elmqvist *et al.* (1964) who concluded that the deficiency in ACh-release is probably not due to an inhibition of ACh synthesis.

Discussion

The experiments reported in this paper enable some preliminary conclusions to be drawn concerning the structural features that determine the affinity of different molecules for the choline transport site and whether or not they are transported. Figure 3 shows the change in affinity resulting from the replacement of one methyl group in tetramethylammonium by alkyl chains of various length. The increment in the affinity constant associated with the addition of a CH_2 group suggests interaction of the alkyl chain with hydrophobic groups in the membrane. The decrease in inhibitory potency that results when a second quaternary nitrogen is added to the molecule is consistent with this idea.

The evidence that these inhibitors interact with hydrophobic groups in the membrane does not necessarily mean that they interact with the lipid part of the membrane. Bergmann & Segal (1954) examined the inhibition of cholinesterase by various mono- and bisquaternary compounds and their results are in many respects very similar to those reported in this paper. They found that within each series, the affinity increased with increasing chain length ; the increment in ΔF associated with the addition of one CH_2 group was -500 cal/mole for the bisquaternary compounds and about -300 cal/mole for monoquaternary compounds. Furthermore, for a similar chain length the monoquaternary compound was bound more strongly than the bisquaternary compound. The affinity constant for pentamethonium, $(Me)_3N\text{-}(CH_2)_5\text{-}N\ (Me)_3$, was $2{\cdot}3 \times 10^2$, that for heptyltrimethylammonium, $CH_3\text{-}(CH_2)_6\text{-}N(Me)_3$, was $1{\cdot}85 \times 10^4$.

The choline- and acetylcholine-binding antibody studied by Marlow, Metcalfe & Burgen (1969) also binds mono- and bisquaternary compounds more firmly the larger the alkyl chain and the CH_2 increment is again about -300 cal/mole. However, with this antibody protein the addition of a second quaternary nitrogen atom seems to have little effect. The affinity constant for pentamethonium is $7{\cdot}25 \times 10^4$, that for the heptyltrimethylammonium $7{\cdot}9 \times 10^4$.

The affinity constant for heptyltrimethylammonium calculated from Fig. 3 is about $6{\cdot}5 \times 10^4$. This means that the affinities of monoquaternary compounds for the choline transport site, the cholinesterase and the choline binding antibody are very similar not only as far as the CH_2 increment is concerned but also with respect to the absolute value. Adding a second quaternary nitrogen to a molecule with a relatively short carbon chain, say heptyl- or octyltrimethylammonium, does not alter significantly its binding to the antibody, reduces its affinity for cholinesterase by two orders of magnitude and its affinity for the choline transport site by three orders of magnitude. As the chain becomes longer the second charged group seems to matter less.

When all four methyl groups in TMA are replaced by ethyl groups—resulting in tetramethylammonium—the affinity for the choline transport site is reduced by a factor of ten (Table 1), possibly because these side chains interfere sterically with the binding of the quaternary nitrogen to a—presumably anionic—site on the carrier. In contrast to this, acetylcholinesterase is more strongly inhibited by TEA than by

TMA (Wilson, 1952) and the affinities of the choline-binding antibody for TMA and TEA are very similar (Marlow *et al.*, 1969).

The break in the curve (Fig. 3) between propyltrimethylammonium and butyltrimethylammonium should be considered together with the evidence that up to propyltrimethylammonium the alkyltrimethylammoniums are transported while butyltrimethylammonium and the larger molecules of this series bind to the transport site but do not enter the cell on the carrier. It is conceivable that the smaller molecules fit on the carrier—accounting for a relatively high affinity constant and the fact that they are transported—while the larger molecules interact not only with the carrier but also with adjacent membrane structures. The fact that the larger molecules of this series are not transported makes it appear unlikely that the "carrier" acts simply by combining with the quaternary nitrogen so that a lipid soluble complex is formed; if this was the case one would expect the added lipid solubility provided by an alkyl chain not to interfere with the movement of the alkyltrimethylammonium-carrier complex across the membrane.

The observation that overnight incubation of erythrocytes with 10^{-5}M DFP substantially reduces the rate of choline transport is unexpected because there is no evidence that the choline transport system has a site that resembles in any way the esteratic site of cholinesterase. It is known that a wide variety of enzymes are inhibited by organophosphorus compounds and Mounter, Alexander, Tuck & Dien (1957) have shown that in the case of chymotrypsin, trypsin, lipase, thrombin and a number of esterases a group of pK 6 to 8 is involved in the reactions between DFP and these enzymes. There is impressive though indirect evidence that this group is an imidazole ring (Cohen & Oosterbaan, 1963). There are no pH and kinetic data on the inhibition of choline transport by DFP and it is therefore not possible to say whether this interaction resembles in any way the interactions between DFP and enzymes.

The affinities of the choline transport site for neuromuscular blocking agents are surprisingly high when compared with its affinities for ganglionic blocking agents. Nevertheless, the concentrations that will block the neuromuscular junction (Paton & Zaimis, 1949) are about two orders of magnitude smaller than those necessary to block choline transport.

REFERENCES

ASKARI, A. (1966). Uptake of quaternary ammonium ions by human erythrocytes. *J. gen. Physiol.*, **49**, 1147–1160.

BERGMANN, F. & SEGAL, R. (1954). The relationship of quaternary ammonium salts to the anionic sites of true and pseudo cholinesterase. *Biochem. J.*, **58**, 692–698.

BIRKS, R. & MACINTOSH, F. C. (1961). Acetylcholine metabolism of a sympathetic ganglion. *Can. J. Biochem. Physiol.*, **39**, 787–827.

BURGEN, A. S. V. & HOBBIGER, F. (1951). The inhibition of cholinesterases by alkylphosphates and alkyl phenol phosphates. *Br. J. Pharmac. Chemother.*, **6**, 592–605.

COHEN, J. A. & OOSTERBAAN, R. A. (1963). The active site of acetylcholinesterase and related esterases and its reactivity towards substrates and inhibitors. In *Handbuch der Experimentellen Pharmakologie*, ed. Koelle, G. B., vol. 15, pp. 299–373. Berlin: Springer-Verlag.

CREESE, R. & MACLAGAN, J. (1967). Autoradiography of decamethonium in rat muscle. *Nature, Lond.*, **215**, 988–989.

CREESE, R. & TAYLOR, D. B. (1967). Entry of labelled carbachol in brain slices of the rat and the action of *d*-tubocurarine and strychnine. *J. Pharmac. exp. Ther.*, **157**, 406–419.

DAHLBÄCK, O., ELMQVIST, D., JOHNS, T. R., RADNER, S. & THESLEFF, S. (1961). An electrophysiological study of the neuromuscular junction in myasthenia gravis. *J. Physiol. Lond.*, **156**, 366–343.

DESMEDT, J. E. (1958). Myasthenic-like features of neuromuscular transmission after administration of an inhibitor of acetylcholine synthesis. *Nature, Lond.*, **182**, 1673–1674.

DESMEDT, J. E. (1966). Presynaptic mechanisms in myasthenia gravis. *Ann. N.Y. Acad. Sci.*, **135**, 209–246.

DIXON, M. & WEBB, E. C. (1964). *Enzymes*, 2nd ed., pp. 325–331. London: Longmans Green & Co., Ltd.

ELMQVIST, O., HOFMAN, W. W., KUGELBERG, J. & QUASTEL, D. M. J. (1964). An electrophysiological investigation of neuromuscular transmission in myasthenia gravis. *J. Physiol., Lond.*, **174**, 417–434.

HOBBIGER, F. W. (1956). Chemical reactivation of phosphorylated human and bovine true cholinesterase. *Br. J. Pharmac. Chemother.*, **11**, 295–303.

HODGKIN, A. L. & MARTIN, K. (1965). Choline uptake by giant axons of *Loligo*. *J. Physiol., Lond.*, **179**, 26–27*P*.

KAUZMAN, W. (1959). Some factors in the interpretation of protein denaturation. *Adv. prot. Chem.*, **14**, 1–63.

LEWIN, L. M. & MARCUS, N. (1965). A bioautographic method for identification of choline and its derivatives. *Analyt. Biochem.*, **10**, 96–100.

MARCHBANKS, R. M. (1968). The uptake of (^{14}C) choline into synaptosomes *in vitro*. *Biochem. J.*, **110**, 533–541.

MARLOW, H. F., METCALFE, J. C. & BURGEN, A. S. V. (1969). The structural features determining the affinity of haptens for an antibody binding acetylcholine. *Mol. Pharmac.*, in the Press.

MARTIN, K. (1968). Concentrative accumulation of choline by human erythrocytes. *J. gen. physiol.*, **51**, 497–516.

MARTIN, K. (1969). Inhibition of choline transport by some neuromuscular blocking agents. *J. Physiol., Lond.*, **200**, 49–50*P*.

MATTHEWS, E. K. (1966). The presynaptic effects of quaternary ammonium compounds on the acetylcholine metabolism of a sympathetic ganglion. *Br. J. Pharmac. Chemother.*, **26**, 552–566.

MORGAN, H. E., REGEN, D. M. & PARK, C. R. (1964). Identifications of a mobile carrier-mediated sugar transport system in muscle. *J. biol. Chem.*, **239**, 369–374.

MOUNTER, L. A., ALEXANDER, H. C., TUCK, K. D. & DIEN, L. T. H. (1957). The pH dependence and dissociation constants of esterases and proteases treated with diisopropylfluorophosphate. *J. biol. Chem.*, **226**, 867–872.

PATON, W. D. M. & ZAIMIS, E. J. (1949). The pharmacological actions of polymethylene bistrimethyl ammonium salts. *Br. J. Pharmac. Chemother.*, **4**, 381–400.

PATON, W. D. M. & ZAIMIS, E. J. (1951). Paralysis of autonomic ganglia by methonium salts. *Br. J. Pharmac. Chemother.*, **6**, 155–158.

STEIN, W. D. (1967). The movement of molecules across cell membranes. *Theoretical and Experimental Biology*, vol. 6. London: Academic Press.

WILBRANDT, W. & ROSENBERG, T. (1961). The concept of carrier transport and its corollaries in pharmacology. *Pharmac. Rev.*, **13**, 109–183.

WILSON, I. B. (1952). Acetylcholinesterase. XII. Further studies of binding forces. *J. biol. Chem.*, **197**, 215–225.

(*Received March* 17, 1969)