On the Mechanism of the Potassium Loss from Brain Slices Induced by Cholinesterase Inhibitors

By K. P. STRICKLAND* AND R. H. S. THOMPSON

Department of Chemical Pathology, Guy's Hospital Medical School, London

(Received 14 October 1954)

A number of reports have appeared suggesting that cholinesterase plays an important role in relation to cell membrane permeability. Rothenberg (1950) and Grundfest & Nachmansohn (1950), for example, have shown that the cholinesterase inhibitors diisopropylphosphorofluoridate (DFP) and eserine in concentrations of about 0.02M increase the exchange of radioactive sodium (^{24}Na) between the axoplasm of giant squid axons and sea water containing the ^{24}Na , and that DFP decreased the exchange of radioactive potassium (^{42}K) under similar conditions. These results were interpreted as showing an increase in ion permeability of the axon, and it was suggested that the cholinesterase system may be important in this connexion.

Work on mammalian erythrocytes (Greig & Holland, 1949; Lindvig, Greig & Peterson, 1951; Greig, Faulkner & Mayberry, 1953) has also led to the conclusion that an active acetylcholine-cholinesterase system is essential to prevent a gain of sodium or a loss of potassium by the erythrocyte. With the cholinesterase uninhibited and adequate amounts of acetylcholine present, the erythrocyte was able to take up potassium or extrude sodium, providing a loss of potassium and a gain of sodium had occurred previously. When the cholinesterase was inhibited (eserine, 10^{-4}M) potassium was lost and sodium was gained. The dependence of the permeability of the erythrocyte on cholinesterase activity is not, however, well established, for Taylor, Weller & Hastings (1952) have presented evidence that potassium loss from the human red cell does not occur until concentrations of eserine (10^{-3}M) and DFP ($2.5 \times 10^{-3}\text{M}$) are reached that are 10 times greater than those necessary to cause complete inhibition of cholinesterase. This is supported by the finding (Thompson & Whittaker, 1952) that concentrations of *p*-nitrophenyl diethyl phosphate (E 600) and DFP, capable of causing 100% inhibition of cholinesterase, have no direct effect on active sodium transport in the human red cell.

Kirschner (1953) has, nevertheless, observed that the active sodium transport across frog's skin at first increased and then sharply decreased when tetraethyl pyrophosphate (TEPP), eserine or

procaine was applied to the inner side of the skin. He also suggested that there may be a correlation between sodium transport and cholinesterase activity since all three compounds are anti-cholinesterases.

Because of these divergent observations and interpretations, and since no further work has been reported in this connexion with nervous tissue, the effects of a number of cholinesterase inhibitors on the loss of potassium from brain slices have been investigated, and an attempt has been made to correlate these effects with inhibition of cholinesterase activity.

Three typical cholinesterase inhibitors have been used for this investigation. They are: (1) DFP, a selective inhibitor of pseudocholinesterase but nevertheless a potent inhibitor of true cholinesterase; (2) eserine, which inhibits both enzymes about equally; and (3) Nu 1250, the synthetic prostigmine analogue *N*-*p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide, which selectively inhibits true cholinesterase and yet is a strong inhibitor of pseudocholinesterase (Hawkins & Mendel, 1949).

A preliminary account of this work has appeared (Strickland & Thompson, 1954).

METHODS

Media

Medium 1. This was a phosphate buffer, pH 7.3, containing 0.011M glucose, described by Dickens & Greville (1935). The Warburg vessels were gassed with O_2 when this medium was used. The medium contained 9.4 mg. K^+ /100 ml.

Medium 2. This was a bicarbonate buffer, pH 7.4, described by Dickens & Greville (1935). This medium (with or without 0.011M glucose) was used for most of the cholinesterase estimations. Vessels were gassed with a 95% N_2 + 5% CO_2 mixture.

Medium 3. A calcium-free phosphate buffer, pH 7.4, containing 0.011M glucose was used (composition of buffer: NaCl , 0.121M; KCl , 0.0026M; NaHCO_3 , 0.0014M; NaH_2PO_4 , 6H $_2\text{O}$, 0.10M; MgCl_2 , 6H $_2\text{O}$, 0.0008M; adjusted to pH 7.4 with 20% (w/v) NaOH). Vessels were gassed with O_2 ; K content = 10.3 mg. K^+ /100 ml.

Medium 4. This was the bicarbonate medium, pH 7.4, used by Ternér, Eggleston & Krebs (1950). This medium

* Postdoctorate Overseas Fellow, National Research Council of Canada.

contained glucose, 0.011M, and L-glutamate, 0.01M. Vessels were gassed with 95% O₂ + 5% CO₂. K content = 23.1 mg. K⁺/100 ml.

Preparation and incubation of tissues

Since it is known that certain cholinesterase inhibitors produce damage to the nervous tissue of the hen resulting in demyelination (Earl & Thompson, 1952; Barnes & Denz, 1953), this species was used for all the experimental work.

Potassium experiments. Hens were killed by decapitation and the cerebral hemispheres quickly removed and slices cut free-hand with a safety-razor blade moistened with the medium. The slices were transferred to a vessel containing 10–12 ml. of the incubating medium to be used (in the case of medium 4 the solution was maintained in equilibrium with 5% CO₂). Slices were gently blotted on filter paper, transferred to tared Warburg vessels containing 2.0 ml. of incubating medium and weighed (weights ranged from 100 to 150 mg.). The vessels were gassed for 7–10 min. with the appropriate gas mixture and then placed in a bath at 38°. After reaching bath temperature (10 min.) the vessels were sealed and the slices incubated for 1 hr. during which time either O₂-uptake or CO₂-evolution was measured. Samples were run in duplicate.

Cholinesterase experiments. Slices were prepared as described above. Homogenates, containing 50–100 mg. of brain slices/ml., were prepared with medium 2 for those experiments in which the ratios of homogenate to slice cholinesterase activities (H/S) were compared. The homogenization was carried out at room temperature for 2–3 min. by rapid up-and-down motion in a glass tube fitted with a ball-ended glass plunger.

Potassium estimations

After incubation, the medium of each sample was pipetted off with a Pasteur pipette, and centrifuged to remove small suspended pieces of tissue. The supernatant medium, if necessary, was diluted with 0.9% NaCl (i.e. 350 mg. Na⁺/100 ml.) to give a solution containing 10–15 mg. K⁺/100 ml. A portion of this solution was diluted 100 times with a dilute NaCl–Li₂SO₄ solution (NaCl, 3.2 × 10⁻³M; Li₂SO₄, 4.4 × 10⁻⁴M). Samples prepared in this way were then flamed against K standards (containing the same Na⁺ and Li⁺ contents) in an internal standard instrument designed and constructed in this laboratory (Baker, 1955). The error in the K method was estimated to be less than ±2%. The K present in the original medium was subtracted from the K in the sample medium and the difference (whether negative or positive) was divided by the dry wt. of the sample. Potassium lost to the medium is expressed as µg. K lost to the medium/mg. dry wt./hr. Dry wt. estimations were done on slices gently blotted, as for the slices used for incubation, and dried at 105°. The mean wet wt./dry wt. ratio for the blotted slices was 7.7. The potassium of the slices was determined as follows: the slices were homogenized in 10% (w/v) trichloroacetic acid, and, after centrifuging, a sample of the supernatant was suitably diluted for estimation of K in the flame photometer. The mean K content of fresh chicken brain was found to be 104.5 m-equiv./kg. (range 90.8–114.4), which agrees well with the values ranging from 90.4 to 110.0 m-equiv./kg. found for guinea pig brain cortex by Turner *et al.* (1950).

Cholinesterase estimations

These were carried out manometrically at pH 7.4 and at 38° using the Warburg technique (Ammon, 1933). All measurements were made in duplicate and were corrected for non-enzymic hydrolysis of the substrate. Incubation times with acetyl- and acetyl-β-methyl-choline as substrates varied from 15 to 30 min. For butyrylcholine all experiments were run for 1 hr. Esterase activity is expressed as µl. CO₂/g. wet wt. tissue/hr.

Substrates

These were acetylcholine perchlorate (ACh) (British Drug Houses Ltd.); acetyl-β-methylcholine chloride (MCh) (Savory and Moore Ltd.); butyrylcholine perchlorate (BuCh) prepared by Dr G. R. Webster (Earl, Thompson & Webster, 1953) and dimethylaminoethyl acetate (DMAEA) prepared in this laboratory by the method of Jones & Major (1930).

The esters were dissolved in bicarbonate buffer (usually medium 2) immediately before use; the final concentrations used throughout were 0.015M for ACh and DMAEA and 0.03M for MCh and BuCh.

Inhibitors

These were eserine sulphate (British Drug Houses Ltd.); diisopropyl phosphorofluoridate (DFP), kindly provided by the Ministry of Supply, Experimental Station, Porton, Wilts; *N*-*p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide (Nu1250) (Hoffman la Roche Ltd.).

The inhibitors were dissolved in buffer immediately before use and added to the main compartment of the Warburg vessel to give the desired final concentration just before the vessels were gassed.

RESULTS

Effect of cholinesterase inhibitors on potassium loss from brain slices

The results of some typical experiments are reported in Table 1. It will be seen that with medium 1, and in the absence of any cholinesterase inhibitor, the K loss to the medium is small (the positive sign indicates a loss of K from the slices to the medium, whereas a negative sign indicates a gain of K by the slices from the medium). Small effects of ±0.5 µg. K loss/mg. dry wt./hr. are not regarded as significant since these values could still be within the possible 2% error in potassium estimations. Medium 3 is less satisfactory for potassium work since there appeared to be a significant loss of K from the uninhibited slices. This medium was used only because it provided a high buffering capacity for the situations indicated in Table 1, where the pH was considerably lowered (to values of less than 6.6) with medium 1 as a result of the high concentrations of the inhibitors used. Potassium analysis of the tissue following incubation in either medium 1 or 3 indicated that the slices had a large potassium deficit (40–50%)

compared to the K content *in vivo*. Therefore, the system (medium 4) described by Turner *et al.* (1950) containing L-glutamate was used. Typical experiments with this medium (Table 1) show that the slices have taken up considerable amounts of K. Potassium analysis of the tissue revealed that the K level was brought up to 85–95% of the *in vivo* concentration. In agreement with Turner *et al.* (1950) these results indicate that medium 4 is the better system to use for potassium studies.

and 3, and significantly reduced the uptake of K at 2×10^{-4} M with medium 4.

The effects of cholinesterase inhibitors on K loss become more obvious from Table 2, in which the extra K loss brought about by the presence of the inhibitor is given. Again, the close parallelism in effects observed with each medium is evident, but, since medium 4 is better for potassium investigations, the effects of the cholinesterase inhibitors cited below are based on the experiments

Table 1. *Typical experiments showing the loss of potassium from brain slices in the presence and absence of various cholinesterase inhibitors*

Slices of chicken brain incubated under aerobic conditions at 38° for 1 hr. in media 1, 3 or 4. Glucose, 0.011 M added to each medium. K in incubating medium estimated by flame photometry.

Inhibitor Inhibitor concn. (M) ...	Medium	K loss to medium (μ g. K/mg. dry wt./hr.)				
		0	2×10^{-5}	2×10^{-4}	2×10^{-3}	2×10^{-2}
DFP	1	+0.90	—	+0.42	+0.60	+7.70*
	3	+1.19	—	+0.79	+0.78	+4.74
	4	-4.97	—	-4.79	-4.89	+1.12
Eserine	1	+0.85	—	+0.55	+0.96	+3.26
	4	-5.67	—	-5.21	-4.91	+2.02
Nu1250	1	0.00	—	+0.27	+4.70*	+6.75
	3	+1.86	—	+1.72	+3.12	+7.05
	4	-3.53	-3.30	-1.75	+3.09	—

+ = loss of K from slices to medium.
 - = gain of K by slices from medium.
 * pH lowered considerably.

Table 2. *Extra potassium loss due to the action of cholinesterase inhibitors on brain slices*

Slices incubated as for experiments shown in Table 1. Results for K loss, expressed as μ g. K/mg. dry wt./hr., are means of the differences in K loss to medium in the presence and absence of the inhibitor. Number of experiments in brackets.

Inhibitor Inhibitor concn. (M) ...	Medium	K loss to medium (μ g. K/mg. dry wt./hr.)			
		2×10^{-5}	2×10^{-4}	2×10^{-3}	2×10^{-2}
DFP	1	—	-0.25 (3)	+0.38 (3)	+5.15 (3)*
	3	—	-0.29 (3)	-0.41 (3)	+4.13 (3)
	4	—	-0.11 (2)	+0.40 (2)	+5.61 (2)
Eserine	1	—	-0.10 (3)	+0.23 (3)	+2.89 (3)
	4	—	+0.18 (2)	+0.57 (2)	+6.82 (2)
Nu1250	1	—	+0.05 (3)	+4.02 (3)*	+5.95 (3)
	3	—	-0.43 (3)	+1.19 (3)	+5.43 (3)
	4	+0.40 (2)	+2.19 (3)	+6.53 (4)	+14.2 (1)

+ = extra loss of K by the slices due to action of inhibitor.
 - = extra uptake of K by the slices due to action of inhibitor.
 * pH considerably lowered.

When the effects of the cholinesterase inhibitors on K loss from slices were determined it was encouraging to find that there was almost complete agreement with the three media. With DFP and eserine a large increase in K loss occurred at 2×10^{-2} M with all media. Nu 1250 caused a significant increase in K loss at 2×10^{-3} M with media 1

done with this medium. Concentrations of 2×10^{-3} M DFP or eserine and 2×10^{-5} M Nu 1250 are probably very near those at which a significant potassium loss does occur, for at concentrations 10 times greater, large K losses from the slices have occurred in each experiment. These data on DFP and eserine agree with Rothenberg's (1950) finding of an in-

crease in the ion permeability of giant squid axons exposed to these inhibitors in concentrations also of the order of 2×10^{-2} M.

Inhibition of cholinesterase in brain slices

Since the three cholinesterase inhibitors were found to cause a K loss from brain slices, it was necessary to know the concentrations of these inhibitors that produced complete inhibition of the cholinesterases (i.e. I_{100} values) in order to establish whether these losses of potassium could be related to abolition of cholinesterase activity. Table 3 shows data on the percentage inhibition of the

these concentrations may be more than adequate to cause a complete inhibition of the enzyme. Nevertheless, the estimated I_{100} values are in every case much less than those concentrations at which a significant loss of potassium is observed.

A few experiments were carried out in which brain slices were incubated under aerobic conditions in medium 4 to which 0.015 M ACh had been added. By this means it was possible to compare the effects of DFP on K loss and on cholinesterase inhibition in the same slices; as in the experiments quoted above, 10^{-5} M DFP, although causing 100% inhibition of cholinesterase activity, produced no extra loss of K.

Table 3. *Percentage inhibition of cholinesterase in brain slices*

Slices of chicken brain incubated in medium 2 with or without 0.011 M glucose. Inhibitors were added to give final concentrations shown. Where glucose was present correction was made for CO_2 evolution due to glycolysis. Number of experiments in brackets.

Substrate Inhibitor concn. (M)	Inhibitor	Percentage inhibition					Estimated I_{100} (M)
		10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	
ACh, 0.015 M (with glucose)	DFP	—	39 (2)	97 (3)	99 (4)	—	10^{-5}
	DFP*	—	28 (1)	96 (1)	100 (1)	—	10^{-5}
	Eserine	—	—	70 (2)	90 (3)	99 (4)	10^{-4}
	Nu 1250	—	93 (2)	96 (3)	98 (5)	—	10^{-5}
MCh, 0.03 M (without glucose)	DFP	—	74 (2)	94 (2)	100 (2)	100 (2)	10^{-5}
	Eserine	—	65 (2)	88 (2)	100 (2)	100 (2)	10^{-5}
	Nu 1250	47 (2)	89 (2)	99 (2)	100 (2)	100 (2)	10^{-6}
BuCh, 0.03 M (without glucose)	DFP	88 (3)	96 (3)	100 (3)	—	—	10^{-6}
	Eserine	—	63 (2)	93 (2)	97 (2)	98 (2)	10^{-4}
	Nu 1250	—	84 (2)	93 (3)	98 (3)	99 (3)	10^{-5}

* Medium 4 used, and estimation carried out under aerobic conditions.

cholinesterases of chicken brain slices by varying concentrations of DFP, eserine and Nu 1250. The approximate I_{100} (M) is given in the final column. The experiments with MCh as substrate for the true cholinesterase and with BuCh as substrate for the pseudocholinesterase were performed in a glucose-free bicarbonate medium. This avoided large corrections for the CO_2 production due to glycolysis. When ACh, the physiological substrate, was used for total cholinesterase activity, glucose was added to the bicarbonate medium to maintain the slices under more physiological conditions. Corrections were made for the CO_2 evolved from tissue glycolysis which varied from 2415 to 2960 $\mu\text{l. CO}_2/\text{g. (wet wt.)}/\text{hr.}$ It can be seen (Table 3) that the total cholinesterase activity (ACh) is virtually completely inhibited by 10^{-5} M DFP or Nu 1250 and 10^{-4} M eserine. Almost complete inhibition of the true cholinesterase (MCh as substrate) is attained by 10^{-5} M DFP or eserine and 10^{-6} M Nu 1250. Pseudocholinesterase (BuCh substrate) is practically completely inhibited by 10^{-6} M DFP, 10^{-4} M eserine and 10^{-5} M Nu 1250. For the three inhibitors, the I_{100} values have been taken well up on the asymptotic region of the inhibition curve so that

Table 4. *Ratios of cholinesterase activities obtained with brain homogenates (H) to those obtained with brain slices (S)*

Slices of chicken brain were incubated in medium 2 with or without 0.011 M glucose. Homogenates containing 50–100 mg. slices/ml. were prepared with medium 2.

H/S for BuCh, 0.03 M (pseudo- ChE activity)	H/S for MCh, 0.03 M (true ChE activity)	H/S for ACh, 0.015 M (total ChE activity)	
		Without glucose	With glucose
1.01	1.60	1.28	1.45
0.87	1.30	1.44	1.80
0.94	1.37	1.29	1.84
1.04	1.38	—	—
1.06	1.34	—	—
1.24	1.25	—	—
Mean	1.03	1.34	1.70

Comparison of the cholinesterase activities of slices (S) and of homogenates of slices (H) (H/S ratios, Table 4) shows that pseudocholinesterase activity (BuCh) is about the same in both, whereas true cholinesterase (MCh) and total cholinesterase activities (ACh) are lower in slices than in homo-

genates. When the slices were better maintained by addition of glucose, the H/S ratio with ACh is further increased. The lower activity of cholinesterase observed in brain slices suggests either that the substrate does not reach all of the cholinesterase in the slices or that there are fewer active centres available. If it is supposed that the extra cholinesterase activity (i.e. true or total) of homogenates indicates that the substrate does not penetrate to all of the cholinesterase in slices, then it is also possible that the cholinesterase inhibitors do not penetrate to all of the enzyme. A number of experiments were therefore carried out in order to clarify this point.

Experiments were designed to determine whether this extra homogenate activity is inhibited in slices first exposed to I_{100} concentrations of inhibitor, washed free from excess, unreacted inhibitor and then homogenized. Since, however, some 'reversal activity' due to re-activation of the enzyme resulting from washing and re-suspension in an inhibitor-free medium is to be expected, particularly in the case of eserine, the activity of the washed I_{100} -treated slices must also be determined. If we assume that the washing lowers the concentration of free inhibitor below the level required to cause significant enzyme inhibition, then penetration of the inhibitor to all the cholinesterase in the slices will cause the extra homogenate activity of the I_{100} -treated slices (i.e. $H_{I_{100}} - S_{I_{100}}$) to be negligible compared with the extra activity of the unpoisoned homogenate (i.e. $H - S$). The results of these experiments are shown in Table 5, from which it can be seen that the extra homogenate activity of the poisoned and washed slices amounts to only 0-12% of the extra activity of the unpoisoned homogenate. We interpret this to mean that 97-100% of the total cholinesterase activity is inhibited in the slice. This was confirmed using MCh as substrate and 10^{-5} M DFP.

Further proof that the I_{100} values are able to inhibit all of the cholinesterase is provided by the results of experiments in which the effect of DFP and eserine on cholinesterase activity was determined using DMAEA as substrate. This tertiary analogue of acetylcholine was used, since it has been reported by Wilson & Cohen (1953) that it penetrates to the 'internal' cholinesterase of crab nerve, whereas ACh only reaches the external enzyme. If it is also true for brain slices that DMAEA reaches all the cholinesterase, then the observation (see Table 6) that 10^{-5} M DFP and 10^{-4} M eserine (i.e. I_{100}) completely inhibit the hydrolysis of DMAEA provides conclusive evidence that these concentrations of DFP and eserine also reach all the enzyme. The additional observation that the ratio of the homogenate cholinesterase activity (H) and the slice cholinesterase activity

Table 5. *The effect of I_{100} concentrations of inhibitors on the extra cholinesterase activity observed with homogenates*

Slices incubated under conditions similar to the experiments described in Table 4. Poisoned slices were suspended in medium containing I_{100} for 15 min. and then washed twice in approx. 10 ml. inhibitor-free media. Activity expressed as μ l. CO_2 /g. (wet wt.)/hr.									
I_{100} (M)	Unpoisoned homogenate (H) of slices	Unpoisoned slices (S)	(H - S)* 0.015 M ACh + 0.011 M Glucose	Homogenate ($H_{I_{100}}$) of poisoned slices	Poisoned slices ($S_{I_{100}}$) (i.e. reversal activity)	($H_{I_{100}} - S_{I_{100}}$)	% of extra homogenate activity not inhibited by I_{100} , i.e. $(H_{I_{100}} - S_{I_{100}}) \times 100 / (H - S)$	% Inhibition of total ChE, i.e. $[H - (H_{I_{100}} - S_{I_{100}})] \times 100 / H$	
DFP, 10^{-5}	16 140	11 620	4520	250	0	250	6	98	
	12 540	8 410	4130	196	0	196	5	98	
DFP, 10^{-6}	10 560	9 120	1440	0	118	- 118	0	100	
	19 830	11 460	8370	2660	2890	- 230	0	100	
Eserine, 10^{-5}	11 560	9 720	1840	3080	2870	210	12	98	
	14 170	10 470	3700	686	419	267	7	98	
Nu1250, 10^{-5}	18 730	10 290	8440	1160	823	337	4	98	
				0.03 M MCh					
DFP, 10^{-5}	6 800	5 230	1570	141	22	119	8	98	
	7 470	4 650	2820	326	70	256	9	97	

* (H - S) = extra homogenate activity. † = medium 4 was used.

(S) is the same when either DMAEA or ACh is used as substrate indicates that in brain slices ACh also must penetrate to all the available enzyme. If this were not true, the H/S ratio for ACh should have been greater than the H/S ratio for DMAEA. Since the H/S ratio is considerably greater than unity for each substrate, homogenization of the tissue has rendered many more enzyme centres active.

potassium from respiring slices of chicken brain. DFP and eserine cause a loss of potassium at concentrations (2×10^{-2} M) similar to those that increase the permeability of giant squid axons (Rothenberg, 1950). A summary of these effects is given in Table 7, where it can be seen that the concentration of DFP or eserine may be increased 200 times above the minimum concentration causing 100 % inhibi-

Table 6. *The effect of DFP and eserine (I_{100}) on cholinesterase activity with DMAEA as substrate*

Slices incubated in medium 4 at 38° under anaerobic conditions. Correction made for CO_2 evolved due to glycolysis. Activity expressed as $\mu\text{l. CO}_2/\text{g. tissue (wet wt.)}/\text{hr.}$

Substrate (M)	Inhibitor (M)	ChE activity of slices (S)	% Inhibition	ChE activity of homogenate (H)	H/S
Expt. 1					
ACh, 0.015	—	11 320	—	21 310	1.88
DMAEA, 0.015	—	3 990	—	7 665	1.92
DMAEA, 0.015	DFP, 10^{-5}	— 317	100	—	—
DMAEA, 0.015	Eserine, 10^{-4}	— 494	100	—	—
Expt. 2					
ACh, 0.015	—	10 600	—	17 200	1.62
DMAEA, 0.015	—	4 410	—	7 275	1.65
DMAEA, 0.015	DFP, 10^{-5}	— 253	100	—	—
DMAEA, 0.015	Eserine, 10^{-4}	— 531	100	—	—

It will be seen from the results given in Table 6 that the addition of DFP or eserine resulted in an apparently negative ChE activity in the presence of DMAEA. This negative value is due to the listed figures being derived by subtraction of the CO_2 due to glycolysis from the observed CO_2 output in the presence of the substrate. Such negative values might therefore arise if the basic CO_2 output due to glycolysis had been markedly inhibited by the DFP or eserine or by the DMAEA. If such had been the case the exact extent to which the ChE had been inhibited would not have been revealed, and it would not therefore be possible to conclude that 100 % inhibition had been effected. To clarify this point, we therefore carried out estimations of lactic acid on trichloroacetic acid filtrates of the contents of the Warburg flasks at the end of the period of CO_2 measurement, using the method of Barker & Summerson (1941). We found that the amount of lactic acid present was unaffected by either ACh or DMAEA, or by DMAEA together with either of the inhibitors. In view of this we feel that we can safely conclude that virtually complete inhibition of the ChE has been achieved, although we are unable to explain the negative values listed for the corrected CO_2 output.

DISCUSSION

These findings indicate that DFP, eserine and Nu1250 in concentrations that are much higher than those required for the complete inhibition of cholinesterase are capable of causing a loss of

Table 7. *Summary of the effects of cholinesterase inhibitors on brain slices*

Inhibitor	Minimum concentration (M) for 100 % inhibition of true cholinesterase	Maximum concentration (M) showing no effect on K^+ loss
DFP	10^{-5}	2×10^{-3}
Eserine	10^{-5}	2×10^{-3}
Nu 1250	10^{-6}	2×10^{-5}

tion of cholinesterase without any effect on the potassium loss from the brain slices. In the case of Nu1250 the concentration may be increased 20 times. Since the cholinesterase activity was practically negligible in a homogenate of slices that had been previously suspended in buffer containing the minimum concentration of inhibitor necessary for 100 % inhibition of cholinesterase, and then washed in inhibitor-free medium, and since I_{100} concentrations completely inhibited cholinesterase activity using DMAEA as substrate, there seems to be little doubt that all of the cholinesterase is reached by these concentrations. The experiments in which both the potassium loss and the cholinesterase inhibition were measured on the same slices provide further proof that much higher concentrations of DFP are required for potassium loss than for complete cholinesterase inhibition. The data, therefore, support Rothenberg (1950) in regard to DFP and eserine being able, in high concentrations, to cause an increase in permeability to potassium,

but do not lend support to his suggestion that inhibition of cholinesterase leads to an increase in the permeability of cell membranes to ions.

Our conclusion that the permeability of the nerve cell to potassium is not increased by cholinesterase inhibition contrasts with that of Greig and colleagues (Greig & Holland, 1949; Lindvig *et al.* 1951; Greig *et al.* 1953) for the erythrocyte. Their work seems to suggest that the erythrocyte requires an active acetylcholine-cholinesterase system to maintain its internal ion environment.

However, in agreement with our findings, a number of workers have concluded that the human erythrocyte does not require an active cholinesterase to maintain ion levels. Christensen & Riggs (1951) indicated that eserine does not cause potassium loss by inhibiting cholinesterase, since concentrations of eserine required to cause a measurable potassium transfer were at least 10 times those required to stop all cholinesterase action in plasma. The observation that part of the cation deficit developed by a loss of potassium from erythrocytes in the presence of $5-50 \times 10^{-3}M$ eserine was accounted for by an uptake of eserine-cation by the cell would suggest that eserine readily penetrates into the cell. Since eserine inhibits both the red cell and plasma cholinesterases about equally, there seems little doubt that the cholinesterase of the red cell would be inhibited by concentrations of eserine much less than those required to cause potassium loss. Taylor *et al.* (1952) and Thompson & Whittaker (1952) have also shown that the human erythrocyte does not require an active cholinesterase to prevent potassium loss or to maintain active sodium transport.

Furthermore, Holland, Dunn & Greig (1952) found that the hydrolysis of ACh increases the permeability of the isolated guinea pig auricle and thus causes a loss of potassium, whereas an inhibition of the enzyme prevents such a loss to a potassium-free Tyrode's solution. They interpreted the results as showing a correlation between permeability and cholinesterase activity that was opposite in nature to the correlation for the erythrocyte.

Measurements of O_2 uptake and of CO_2 evolution by brain slices, which we are hoping to describe later, suggest that the potassium loss induced by these anti-cholinesterases may be associated with an effect of these substances on the energy-yielding mechanisms of the cells. Such a relationship would accord with the results of Dixon (1949) and Turner *et al.* (1950). Dixon (1949) found that glycolysis was essential to prevent a further loss of potassium from slices of rabbit brain, while Turner *et al.* (1950) have shown that aerobic conditions and the substrates glucose and L-glutamate are essential in a bicarbonate-saline medium before potassium is taken up by slices of guinea pig brain cortex. Again, if the

analogy with the erythrocyte is considered, Danowski (1941), Harris (1941), Maizels (1949), Flynn & Maizels (1950) and Ponder (1950), have found glycolysis and hence energy to be necessary for the replacement of potassium in human erythrocytes.

It would be unfair to discuss the implications that the dissociation of ion permeability from cholinesterase activity may have on an interpretation of various processes related to or dependent on potassium in the intact brain without some confirmation *in vivo*. These results only serve to indicate that cholinesterase activity is not involved in the prevention of the loss of measurable amounts of potassium from slices of chicken brain. For technical reasons it is, of course, not easy to determine the point at which complete inhibition of an enzyme occurs, and it is possible that a very small level of cholinesterase activity may still be present under conditions which we have interpreted as showing complete or virtually complete inhibition. On the other hand, it is known that conduction in nerve fibres is blocked (Wilson & Cohen, 1953), and manifestations of acetylcholine accumulation in other organs occur, when only 80% inhibition of cholinesterase has been brought about (Mazur & Bodansky, 1946; Koelle & Gillman, 1946; Hawkins & Mendel, 1947; Nachmansohn & Feld, 1947). In view of this it seems unlikely that the very low levels of cholinesterase activity, which might still be present in our estimates of 98-100% inhibition, would be sufficient to maintain the normal ionic balance across the nerve cell membrane, if this property were really dependent on and determined by an active cholinesterase. It may be that more refined methods could show a relation between these two processes, which if true must involve very small amounts of potassium (e.g. the amounts involved in the depolarization of axons). Bearing these limitations in mind, however, it seems likely that the overall maintenance of ion levels, and hence the permeability of the cell membrane, is largely dependent on the energy-supplying mechanisms of the cell rather than on an adequate acetylcholine-cholinesterase system.

SUMMARY

1. The anti-cholinesterases (1) diisopropyl phosphorofluoridate (DFP), (2) eserine and (3) Nu1250 (a prostigmine analogue) when present in concentrations of $2 \times 10^{-3}M$, $2 \times 10^{-2}M$ and $2 \times 10^{-4}M$, respectively, cause a loss of potassium from respiring brain slices.

2. The loss of potassium does not correlate with the anti-cholinesterase properties of these inhibitors, since a virtually complete inhibition of the cholinesterase in the slices occurred with concentrations of

DFP, eserine and Nu 1250 that were, respectively, 200, 200 and 20 times less than those concentrations just capable of causing a potassium loss.

3. It seems that the permeability of the nerve cell to potassium is dependent on the energy-producing reactions of the cell rather than on the presence of an active acetylcholine-cholinesterase system.

We wish to thank the Medical Research Council for a grant towards the expenses of this work. Our thanks are also due to Mr D. E. Jarvis for technical assistance.

REFERENCES

- Ammon, R. (1933). *Pflüg. Arch. ges. Physiol.* **233**, 486.
 Baker, R. W. R. (1955). *Biochem. J.* **59**, 566.
 Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
 Barnes, J. M. & Denz, F. A. (1953). *J. Path. Bact.* **65**, 597.
 Christensen, H. N. & Riggs, T. R. (1951). *J. biol. Chem.* **193**, 621.
 Danowski, T. S. (1941). *J. biol. Chem.* **139**, 693.
 Dickens, F. & Greville, G. D. (1935). *Biochem. J.* **29**, 1468.
 Dixon, K. C. (1949). *Biochem. J.* **44**, 187.
 Earl, C. J. & Thompson, R. H. S. (1952). *Brit. J. Pharmacol.* **7**, 685.
 Earl, C. J., Thompson, R. H. S. & Webster, G. R. (1953). *Brit. J. Pharmacol.* **8**, 110.
 Flynn, F. & Maizels, M. (1950). *J. Physiol.* **110**, 301.
 Greig, M. E., Faulkner, J. S. & Mayberry, T. C. (1953). *Arch. Biochem. Biophys.* **43**, 39.
 Greig, M. E. & Holland, W. C. (1949). *Arch. Biochem.* **23**, 370.
 Grundfest, H. & Nachmansohn, D. (1950). *Fed. Proc.* **9**, 53.
 Harris, J. E. (1941). *J. biol. Chem.* **141**, 579.
 Hawkins, R. D. & Mendel, B. (1947). *Brit. J. Pharmacol.* **2**, 173.
 Hawkins, R. D. & Mendel, B. (1949). *Biochem. J.* **44**, 260.
 Holland, W. C., Dunn, C. E. & Greig, M. E. (1952). *Amer. J. Physiol.* **168**, 546.
 Jones, L. W. & Major, R. T. (1930). *J. Amer. chem. Soc.* **52**, 307.
 Kirschner, L. B. (1953). *Nature, Lond.*, **172**, 348.
 Koelle, G. B. & Gillman, A. (1946). *J. Pharmacol.* **87**, 421.
 Lindvig, P. E., Greig, M. E. & Peterson, S. W. (1951). *Arch. Biochem.* **30**, 241.
 Maizels, M. (1949). *J. Physiol.* **108**, 247.
 Mazur, A. & Bodansky, O. (1946). *J. biol. Chem.* **163**, 261.
 Nachmansohn, D. & Feld, E. A. (1947). *J. biol. Chem.* **171**, 715.
 Ponder, E. (1950). *J. gen. Physiol.* **33**, 745.
 Rothenberg, M. A. (1950). *Biochim. biophys. Acta*, **4**, 96.
 Strickland, K. P. & Thompson, R. H. S. (1954). *Biochem. J.* **58**, xx.
 Taylor, I. M., Weller, J. M. & Hastings, A. B. (1952). *Amer. J. Physiol.* **168**, 658.
 Terner, C., Eggleston, L. V. & Krebs, H. A. (1950). *J. Biochem.* **47**, 139.
 Thompson, E. H. & Whittaker, V. P. (1952). *Biochim. biophys. Acta*, **9**, 700.
 Wilson, I. B. & Cohen, M. (1953). *Biochim. biophys. Acta*, **11**, 147.

The Fractionation of Mammalian Serum Proteins with Ether

By MARGARET E. MACKAY

Medical Research Council External Scientific Staff, Lister Institute, London

(Received 12 January 1955)

A technique for the separation of the protein components of human citrated plasma using aqueous systems containing ether has been described by Kekwick & Mackay (1954). When blood protein fractions from animals have been required the method devised for human plasma was used for the fractionation of their serum proteins. Because of the diversity of the electrophoretic pattern of animals' sera, not only from species to species, but between individuals of the same species, the consistency of the composition of the fractions separated was a little surprising, and as the results to be described show, the method may have a wider application than was at first envisaged.

MATERIALS

Serum. The serum of guinea pigs, rats and rabbits was prepared from the pooled blood of groups of 10–250 animals. These animals were bled by cutting the throat of the animal

after it had been stunned. Ox, sheep and monkey blood also represented a pool from several animals, while that of cat, dog and horse was taken from a single animal by cannula from a vein, when necessary under an anaesthetic. The blood was collected in dry bottles, allowed to clot at room temperature, and stored overnight at 4°. Serum was collected after the blood had been centrifuged at 1500 rev./min. for 30 min. at 0°.

Diethyl ether. This was of anaesthetic grade, free from antiperoxidants and was stored in the dark at 4°.

Ethanol. Rectified spirit was diluted with water to 50% (v/v) alcohol.

Acetate-phosphate buffers: Na_2HPO_4 , $2\text{H}_2\text{O}$, 0.5 M = 89.04 g./l.; acetic acid, 0.5 M. 2.0 M acetic acid is diluted 1 vol. to 4 and is standardized by titration. 2.0 M acetic acid (114.5 ml. glacial acetic acid/l.) was standardized by diluting to approximately 0.2 M and titrating against standard 0.2 M-NaOH. (a) pH 4.0: 0.5 M-HAc: 86 ml./l. = 0.034 M; 0.5 M- Na_2HPO_4 , $2\text{H}_2\text{O}$: 14 ml./l. = 0.007 M; $I = 0.05$. (b) pH 6.0: 0.5 M-HAc: 46 ml./l. = 0.023 M; 0.5 M- Na_2HPO_4 , $2\text{H}_2\text{O}$: 54 ml./l. = 0.027 M; $I = 0.05$. (c) pH 7.0: Na_2HPO_4 ,