peptide material ('fibrinopeptide') from acetyl-fibrinogen can be demonstrated; the ultracentrifuge data show definitely that some of the heavier intermediate polymers are formed in the reaction and that only the 'gel' stage of clotting is completely inhibited. This suggests that the site of attachment of thrombin to fibrinogen is on the bulk part of the molecule and not on the peptide portion split off by thrombin. The strong adsorption of thrombin on fibrin leads to the same conclusion.

SUMMARY

1. Fibrinogen was acetylated under mild conditions at varying pH, giving materials of varying degrees of substitution very similar in physico-chemical properties to the native protein.

2. The clotting properties of the protein are modified by the degree of substitution. The material is no longer clottable by thrombin when the number of free amino groups substituted exceeds 35 %.

3. Acetylated fibrinogen inhibits the reaction between thrombin and fibrinogen. This inhibition appears to be competitive.

4. Although the acetylated fibrinogen does not form a clot when acted upon by thrombin, a peptide is split off and soluble polymers are formed.

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The Effects of Anticholinesterases on Oxidative Phosphorylation in Brain, and their Relation to Potassium Loss and Nerve Degeneration

By K. P. STRICKLAND,* R. H. S. THOMPSON AND G. R. WEBSTER
Department of Chemical Pathology, Guy's Hospital Medical School, London

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A number of reports (Rothenberg, 1950, for the squid axon; Greig, Faulkner & Mayberry, 1953, for the mammalian erythrocyte; Kirschner, 1953, for frog skin) indicating the possible importance of cholinesterase in relation to cell-membrane permeability led to an investigation into the effects of cholinesterase inhibitors on potassium loss from brain slices (Strickland & Thompson, 1955). From this work it was concluded that cholinesterase was not directly concerned with the prevention of the loss of measurable amounts of potassium from slices of chicken brain; thus in each case the cholinesterase inhibitors DFP (diisopropyl phos-

phorofluoridate), eserine and Nu 1250 (the synthetic prostigmine analogue, *N*-*p*-chlorophenyl-*N*-methyl carbamate of *m*-hydroxyphenyltrimethylammonium bromide) caused potassium loss only when a concentration several times greater than that necessary for complete inhibition of cholinesterase activity was reached. Some evidence suggested that these inhibitors caused potassium loss because of their action on the energy-producing reactions of the cell.

In addition to this work on potassium loss, Barnes & Denz (1953) and Cavanagh (1954) have shown that there is a marked similarity between the degenerative changes induced in peripheral nerves of the hen by anticholinesterases such as

* Postdoctorate Overseas Fellow, National Research Council of Canada.

DFP, mipafox (*NN'*-diisopropylphosphorodiamidic fluoride) and TOCP (tri-*o*-cresyl phosphate) and those caused by thiamine deficiency. From the nature of the biochemical changes in thiamine deficiency (Peters, 1936) it appears that the nerve lesions, in this case, result from a block in the energy-yielding reactions of the neurone by the inhibition of pyruvate oxidation.

The possibility that both potassium loss and demyelination induced by anticholinesterases are the result of an inhibition of steps concerned with the production of energy has given rise to the work reported here. More complete observations are presented which show that those concentrations of cholinesterase inhibitors capable of causing potassium loss from brain slices also have some effect on the respiratory processes of the cell (i.e. O_2 uptake or CO_2 evolution). Since energy for the maintenance of the cell is supplied by means of energy-rich phosphate bonds, the effects of several cholinesterase inhibitors on the oxidative phosphorylation of homogenates of chicken brain have been measured. The inhibitors mipafox, TEPP (tetraethyl pyrophosphate) and DPDA (*NN'*-diisopropylphosphorodiamidic anhydride) have been used in addition to DFP, eserine and Nu 1250, already mentioned. Of these inhibitors, DFP and mipafox are capable of inducing demyelination in chickens (Barnes & Denz, 1953). If the demyelination induced by DFP and mipafox is due primarily to a biochemical lesion of the energy-yielding reactions, then it might be expected that these demyelinating compounds should show a more marked effect on oxidative phosphorylation than the non-demyelinating compounds.

METHODS

Experiments relating potassium loss and gas measurements

The preparation of slices of chicken brain, the incubation procedure, the estimation of potassium and the measurement of O_2 uptake and net CO_2 evolved were all carried out as described previously (Strickland & Thompson, 1955).

The media used were as follows:

Medium 1. The phosphate medium, pH 7.3, containing 0.011 M glucose, described by Dickens & Greville (1935).

Medium 3. The calcium-free phosphate medium, pH 7.4, containing 0.011 M glucose, used by Strickland & Thompson (1955).

Medium 4. The bicarbonate medium, pH 7.4, of Ternier, Eggleston & Krebs (1950) containing 0.011 M glucose and 0.01 M L-glutamate.

Oxidative phosphorylation

The system used for measuring oxidative phosphorylation was essentially that described by Case & McIlwain (1951).

Preparation of tissue. Hens were decapitated and the cerebral hemispheres rapidly removed and weighed. A homogenate of 1 g. of brain in 4 ml. of a 0.6% (w/v) KCl + 0.5% (w/v) NaF solution was prepared at 0° as described by Webster (1954).

Incubation. Into the main compartment of each Warburg vessel were placed 2.0 ml. of a medium having a composition such that on dilution to 3.0 ml. by addition of inhibitors, distilled water and the homogenate, the final concentration of each of the components was: glucose, 0.01 M; adenosine triphosphate (Na salt), 0.001 M; adenosine-5' phosphate (K salt), 0.001 M; sodium fumarate, 0.0033 M; glycylglycine buffer (pH 7.4), 0.083 M; KH_2PO_4 - K_2HPO_4 buffer (pH 7.4), 0.0017 M; NaF (from homogenate), 0.022 M, and KCl (from homogenate), 0.013 M; $MgCl_2 \cdot 6H_2O$, 0.017 M. The homogenate (0.6 ml.) was pipetted into the side arm. Strips of filter paper were dipped into 0.2 ml. of 20% KOH solution contained in the centre well. The vessels were gassed for 3 min. with O_2 and then placed in the water bath at 38°. After 10 min. temperature equilibration the homogenate was tipped into the main compartment. The manometers were sealed and the O_2 uptake was measured. After incubation for 0 or 20 min., 1 ml. of the reaction mixture was quickly transferred to 4 ml. of ice-cold 6.25% (w/v) trichloroacetic acid. The protein precipitate was filtered off at 0°.

Estimation of inorganic phosphate. Inorganic orthophosphate was estimated according to the method described by Ernster, Zetterström & Lindberg (1950). The following procedure was adopted to avoid breakdown of acid-labile phosphate esters. The reagents for each analysis (5.0 ml. of isobutanol-benzene 1:1, by vol.; 3.5 ml. of 5% (w/v) trichloroacetic acid and 0.5 ml. of 60% (w/v) perchloric acid) were cooled to just below 0°. A sample (1 ml.) of the clear filtrate was added to the cold reagents, followed by 0.5 ml. of 10% (w/v) ammonium molybdate solution. After standing in stoppered tubes for 1 min., the tubes were shaken vigorously for 30 sec. A portion (3 ml.) of the isobutanol-benzene layer, containing the phosphomolybdic acid complex, was diluted to 9 ml. with acid-ethanol (32 ml. of conc. H_2SO_4 /l. of absolute ethanol) and the blue colour was developed by addition of 0.5 ml. of a dilute $SnCl_2$ solution (1 ml. of stock solution of 10 g. of $SnCl_2 \cdot 2H_2O$ /25 ml. of conc. HCl, diluted to 200 ml. with $N-H_2SO_4$). After colour development for 30 min., the colour intensity was read in a spectrophotometer at 730 m μ .

Inhibitors

Aqueous solutions of the inhibitors were prepared immediately before use. The cholinesterase inhibitors were DFP; DPDA, supplied by the Ministry of Supply, Experimental Station, Porton, Wilts.; mipafox, obtained from the M.R.C. Toxicology Research Unit, Carshalton, Surrey; eserine sulphate (British Drug Houses Ltd.); Nu 1250 (Hoffmann-La Roche and Co.) and TEPP (Albright and Wilson Ltd.).

RESULTS

The effects of DFP on the loss of potassium and on the cholinesterase activity (with acetylcholine as substrate) in the same slices under aerobic conditions are shown in Table 1. It will be seen that in the presence of acetylcholine a significant potassium

loss occurs only when this substrate is hydrolysed sufficiently (after 60 min.) to lower the pH. Hydrolysis of acetylcholine for a shorter period (30 min.) has no effect on potassium loss. At 10^{-5} M DFP, no potassium loss occurs in either the absence or presence of acetylcholine, although there is a complete inhibition of cholinesterase activity. A large loss of potassium becomes noticeable after increasing the concentration of DFP to 2×10^{-2} M. The experimental conditions made it possible to determine the effects of DFP on potassium loss and on cholinesterase activity in the same slices. The results augment the findings of Strickland & Thompson (1955) and lend further support to the conclusion that an increase in the permeability to potassium is not directly associated with an inhibition of cholinesterase activity.

The action of DFP, eserine and Nu 1250 on O_2 uptake and net CO_2 evolution (i.e. CO_2 evolved in excess of O_2 taken up, calculated as $\Delta p \times K_{CO_2}$, when the gas phase is $O_2 + CO_2$, 95:5) has been measured in the experiments in which the effects of these inhibitors on potassium loss from slices of chicken brain have already been reported (Strickland & Thompson, 1955). The results, expressed as means of the percentage inhibitions, are listed in Table 2. The negative sign indicates a stimulation in either uptake or production of the gas measured. Means of the extra potassium loss due to the action of these inhibitors have been included in order to show more easily the relation between extra potassium loss and percentage inhibition of the gas taken up or evolved. In general, some effect on either O_2 uptake or net CO_2 evolution is observed

Table 1. *Effect of DFP on the loss of potassium from brain slices in the presence and absence of acetylcholine (0.015 M ACh) and the corresponding cholinesterase inhibition under aerobic conditions*

Slices incubated under aerobic conditions in the bicarbonate medium, pH 7.4, used by Turner *et al.* (1950), i.e. medium 4 of Strickland & Thompson (1955). K^+ in incubating medium was estimated by flame photometry. K loss expressed as $\mu g.$ of K/mg. dry wt./hr. Gas evolved in the absence of ACh was subtracted from the gas evolved in the presence of ACh to give a measure of the cholinesterase activity. No. of experiments in brackets. + = extra loss of K by the slices in the presence of ACh and/or DFP; - = extra uptake of K by the slices in the presence of ACh and/or DFP.

DFP concn. (M)	Incubation period (min.)	Mean change in K loss relative to slices incubated in absence of ACh and DFP		Inhibition of cholinesterase activity (%)
		No ACh	ACh present	
0	30	0	+0.39 (2)	0
0	60	0	+4.54 (3)*	0
10^{-5}	60	-0.71 (4)	-0.41 (4)	100 (5)
10^{-2}	60	+1.30 (2)	-0.11 (2)	100 (2)
2×10^{-2}	60	+5.87 (2)	+5.72 (2)	100 (3)

* Owing to the high hydrolysis rate of ACh the pH decreased to about 6.5.

Table 2. *Effect of cholinesterase inhibitors on potassium loss, oxygen uptake and CO_2 evolution by slices of chicken brain*

Slices incubated as in the experiments reported in Table 2 of Strickland & Thompson (1955). Results for K loss, expressed as $\mu g.$ of K/mg. dry wt./hr., are means of the differences in K loss to the medium in the presence and absence of the inhibitor. Percentage inhibition = $\frac{\text{gas measured in presence of inhibitor}}{\text{gas measured in absence of inhibitor}} \times 100$. The gas measured varied, depending on the medium used. No. of experiments in brackets. A negative sign indicates an increased uptake of K or O_2 or an increased evolution of CO_2 .

Inhibitor	Medium	Gas measured	Inhibitor concn. (M)							
			2×10^{-5}		2×10^{-4}		2×10^{-3}		2×10^{-2}	
			K loss	Inhibition (%)	K loss	Inhibition (%)	K loss	Inhibition (%)	K loss	Inhibition (%)
DFP	3	O_2 uptake	—	—	-0.29 (3)	7 (3)	-0.41 (3)	6 (3)	4.13 (3)	31 (3)
	4	Net CO_2 evolution	—	—	-0.11 (2)	-27 (2)	0.40 (2)	-42 (2)	5.61 (2)	-390 (2)
Eserine	1	O_2 uptake	—	—	-0.10 (3)	3 (3)	0.23 (3)	-7 (3)	2.89 (3)	-7 (3)
	4	Net CO_2 evolution	—	—	0.18 (2)	-4 (2)	0.57 (2)	-13 (2)	6.82 (2)	-81 (2)
Nu 1250	3	O_2 uptake	—	—	-0.43 (3)	24 (3)	1.19 (3)	55 (3)	5.43 (3)	87 (3)
	4	Net CO_2 evolution	+0.41 (2)	-18 (2)	2.19 (3)	-23 (3)	6.53 (4)	-240 (4)	14.2 (1)	—

whenever a potassium loss has occurred. The data on CO₂ evolution are only approximate, since small differences in the manometer readings produce relatively large percentage differences because the CO₂ evolution from the uninhibited sample is not great (e.g. 400 μl. of CO₂/g. of wet wt. tissue/hr.). DFP at 2 × 10⁻² M, where a large potassium loss occurs, causes a 31 % inhibition of O₂ uptake and a 390 % increase in CO₂ evolution. Eserine appears to have no significant effect on respiration, but at 2 × 10⁻² M, where a large potassium loss occurs, it

generates of guinea-pig brain. This system, as expected, responded well to the now classical action of 2:4-dinitrophenol (DNP) as an uncoupler of oxidative phosphorylation. At 10⁻⁶, 10⁻⁵ and 10⁻⁴ M DNP, oxidative phosphorylation, as measured by P/O ratios, was inhibited 18, 73 and 114 % respectively. By using this system, the effects of a number of cholinesterase inhibitors on the P/O ratios were measured. The results of these experiments are shown in Table 3. The percentage inhibition of oxygen uptake, of inorganic phosphate

Table 3. *Effect of cholinesterase inhibitors on respiration and oxidative phosphorylation of homogenates of normal chicken brain*

Reaction mixture 3.0 ml. Final concentrations of reactants as described under Methods. 0.6 ml. of homogenate prepared from 1 g. of cerebral hemispheres/4.0 ml. of ice-cold solution containing 0.6 % KCl and 0.5 % NaF added to side arm and then tipped into main compartment. Incubation at 38° for 20 min. under aerobic conditions. Reaction stopped with trichloroacetic acid.

Inhibitor	Concn. (M)	% Inhibition of					
		O ₂ uptake		P esterification		P/O ratio	
		Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
DFP	10 ⁻⁴	—	0	—	3	—	1
	10 ⁻³	21	11	8	15	-15	5
	10 ⁻²	25	28	63	63	51	48
Eserine	10 ⁻⁴	—	-1	—	2	—	2
	10 ⁻³	0	10	12	7	10	-3
	10 ⁻²	13	25	32	46	18	28
Nu 1250	10 ⁻⁵	-12	5	-2	7	8	2
	10 ⁻⁴	-3	-11	-2	11	0	19
	10 ⁻³	9	28	93	*104	92	*105
	10 ⁻²	45	—	*112	—	*120	—
Mipafox	10 ⁻⁴	2	8	-1	-2	-3	-10
	10 ⁻³	-2	6	-8	5	-6	0
	10 ⁻²	16	14	11	18	-6	6
DPDA	10 ⁻⁴	-2	4	-10	-2	-9	3
	10 ⁻³	-3	-1	2	3	5	3
	10 ⁻²	5	8	60	51	58	46
TEPP	10 ⁻⁴	-7	-2	12	-5	17	-3
	10 ⁻³	-14	-1	-22	0	-8	1
	10 ⁻²	26	37	43	49	22	20

* Inhibitions of more than 100 % indicate breakdown of ester phosphates.

increases by 81 % the CO₂ evolved from medium 4. Inhibition of respiration by Nu 1250 becomes evident at 2 × 10⁻⁴ M where potassium loss is first noted with medium 4. Respiration is strongly inhibited (55 %) and CO₂ evolution very much increased (240 %) at a concentration (2 × 10⁻³ M) of Nu 1250 that produces a large potassium loss. The inhibition of respiration increases to 87 % at 2 × 10⁻² M where larger losses of potassium also occur.

The P/O ratios, i.e. (P atoms esterified)/(O atoms taken up), for homogenates of chicken brain, incubated in the absence of inhibitor, varied from 0.54 to 0.85. These values are within the range reported by Case & McIlwain (1951) for homo-

esterified and of the P/O ratios caused by DFP, eserine, Nu 1250 (the three inhibitors used in the experiments on potassium loss), mipafox, DPDA and TEPP are given for two experiments. DFP has only a small inhibitory effect on O₂ uptake (25–28 %) even at a concentration of 10⁻² M. P esterification, on the other hand, is inhibited 63 %, so that the P/O ratio is inhibited 50 %. Eserine also has only a small effect at 10⁻² M. Nu 1250, however, showed a more striking effect, for at 10⁻³ M P esterification and the P/O ratio were almost completely inhibited, whereas O₂ uptake was only slightly inhibited. It will be seen that in the concentrations used mipafox has no significant effect on the P/O ratio, although at a

concentration of $10^{-2}M$ it causes approximately 15 % inhibition of O_2 uptake and of P esterification. DPDA, even at $10^{-2}M$, has no effect on O_2 uptake, but this concentration inhibits P esterification by 56 % and the P/O ratio by 52 %. TEPP begins to have some effect at $10^{-2}M$, when O_2 uptake, P esterification and the P/O ratio are inhibited 32, 46 and 21 % respectively. In the case of DFP, eserine and TEPP, somewhat higher concentrations are required to produce an inhibition of O_2 uptake than those reported by Brooks, Ransmeier & Gerard (1949) for frog brain. Similarly, $10^{-2}M$ DFP, which causes a 50 % inhibition of oxidative phosphorylation, is 20 times greater than the concentration of DFP that Abood & Gerard (1952) found to cause a complete inhibition of oxidative phosphorylation by rat brain.

DISCUSSION

The results of Strickland & Thompson (1955) and those listed here (Table 1) indicate that inhibition of cholinesterase activity does not detectably affect the overall permeability of the nerve cell to potassium. However, the anticholinesterases DFP, eserine and Nu 1250 each cause a potassium loss at concentrations considerably greater than those required for complete inhibition of cholinesterase. The data in Table 2 suggest that this potassium loss is due to some action on the energy-producing mechanisms of the cell. In every case where a potassium loss has been induced, either O_2 uptake has been inhibited or the CO_2 evolution has increased. This increase in CO_2 evolution could occur either by a stimulation of aerobic glycolysis or by an inhibition of O_2 uptake. Either effect indicates some disruption in the normal metabolism of the cell.

On extending the investigation to include measurements of the effect of these cholinesterase inhibitors on phosphorylation and O_2 uptake by homogenates of chicken brain, an even closer relation is established between potassium loss and a defect in the energy production of the cells of the brain. For the three anticholinesterases used in the study on potassium loss, the inorganic phosphate esterified is inhibited significantly more than the oxygen uptake by concentrations of inhibitors approaching those that cause potassium loss from brain slices. This would seem to indicate more conclusively that these compounds have caused a potassium loss by inhibiting phosphorylation, a step that is essential in the formation of energy-rich phosphate bonds.

The data for DFP disagree with those of Abood & Gerard (1952) for rat brain. They found that $5 \times 10^{-4}M$ DFP produced complete abolition of oxidative phosphorylation. There seems to be no

obvious explanation for this disagreement, but species differences and the use of a different technique by Abood & Gerard (1952) for measurement of the inorganic phosphate esterified may have had some influence.

The findings from the experiments on oxidative phosphorylation do not help in the explanation of the demyelination induced by certain anticholinesterases. No differential effect on oxidative phosphorylation is observed between the cholinesterase inhibitors producing demyelination (DFP and mipafox) and those not producing this lesion (eserine, Nu 1250, DPDA and TEPP). Furthermore, the inhibition of oxidative phosphorylation noted with DFP occurs at a much higher concentration than could possibly exist in chickens paralysed by injection of this compound. Although tri-*o*-cresyl phosphate (a demyelinating anticholinesterase) was not studied, these results fit in well with the observation by Earl, Thompson & Webster (1953) that TOCP in concentrations capable of completely inhibiting pseudocholinesterase and inducing demyelination has no effect either *in vitro* or *in vivo* on glucose or pyruvate oxidation. Despite the histological similarity in the demyelination induced by DFP and mipafox and that caused by thiamine deficiency (Barnes & Denz, 1953) it would seem that some other processes or enzymic mechanisms must be involved in the demyelination produced by these inhibitors. If, as seems likely, a particular enzyme is involved, then it is quite possible that this enzyme will be similar to pseudocholinesterase, an enzyme that is inhibited by all of the demyelinating organophosphorus compounds (see Davison, 1953; Cavanagh & Thompson, 1954). Such an unknown enzyme might be an esterase or phosphatase intimately concerned with either the synthesis or degradation of myelin.

SUMMARY

1. The effect of diisopropyl phosphorofluoridate (DFP), eserine and Nu 1250 (a prostigmine analogue) on O_2 uptake and CO_2 evolution by slices of chicken brain has been related to the potassium loss from the slices induced by these compounds. In every case when potassium loss has occurred, either O_2 uptake has been inhibited or CO_2 evolution has been stimulated.

2. Concentrations of DFP, eserine and Nu 1250, similar to those which produce potassium loss from slices, were found to cause an inhibition of oxidative phosphorylation (as measured by P/O ratios) of homogenates of chicken brain.

3. In addition to these inhibitors, the effects of *NN'*-diisopropylphosphorodiamidic fluoride (mipafox), *NN'*-diisopropylphosphorodiamidic anhy-

dride and tetraethyl pyrophosphate on oxidative phosphorylation have also been investigated.

4. The inhibition of oxidative phosphorylation obtained with the six anticholinesterases is discussed in relation to the degenerative changes which certain of these compounds produce in the nervous system.

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The Respiration of Arterial Tissue

BY ANNE E. HENDERSON AND J. D. B. MACDOUGALL

*Departments of Biochemistry and Anatomy, Queen's College, Dundee
(University of St Andrews)*

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The degenerative diseases affecting arteries have received a great deal of attention, and much work, both clinical and experimental, has been done on such problems as the pathogenesis of atherosclerosis. By comparison, little is known about the metabolism of the normal artery. The histological appearances of the artery do not suggest that it is a particularly active tissue, but the presence of *vasa vasorum* (i.e. the nutrient vessels of the artery wall) in the larger vessels does indicate that a moderate amount of respiratory exchange might be expected. This expectation has been partly confirmed for the rat, both by Lazovskaya (1943) and by Briggs, Chernick & Chaikoff (1949). As these reports, however, are somewhat conflicting, it was decided to reinvestigate the respiration of the rat aorta and to extend the work to include several other species. In addition, the respiratory activity of arterial tissue during the development of the embryo chick was studied, and the results of this work were compared with changes observed in the histological appearance of the arteries during the same period of embryological life.

MATERIALS AND METHODS

Respiration method I. The direct method of Warburg was used throughout for estimating the oxygen uptake of tissues whose dry weights were over 20 mg. Krebs solution 'Medium II' was used as the respiration medium (Krebs, 1950).

The laboratory animals were killed by stunning followed by severance of the vertebral column. A dorsal approach was used to expose the aorta, which was rapidly excised and placed in a dish containing ice-cold Krebs solution surrounded by powdered ice. The blood contained in the aorta was immediately removed by washing through with the Krebs solution by means of a syringe, and any blood and fat adhering to the outer wall were also removed. In the experiments carried out on slaughterhouse material, a portion of aorta was placed in a bottle containing the ice-cold Krebs solution, surrounded by ice in a vacuum flask and quickly transported to the laboratory. The measurement of oxygen uptake was carried out on artery rings, approx. 2 mm. in length, so that although the thickness of the artery wall varied in the different species used, the depth of tissue in the rings was relatively constant. After equilibration, the oxygen uptake was measured for 45 min., during which period it was found to be linear.