Biochemical Pharmacology, 1965, Vol. 14, pp. 237–244. Pergamon Press Ltd., Printed in Great Britain.

THE EFFECTS OF NEUROMUSCULAR BLOCKING AGENTS ON MITOCHONDRIA—II.

EFFECTS OF D-TUBOCURARINE, PYRROLIZIDINE ALKALOIDS AND OF COMPLEX IONS ON SWELLING OF MITOCHONDRIA

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(*Received* 22 *May* 1964; *accepted* 13 *October* 1964)

Abstract—The alkaloids, D-tubocurarine, lasiocarpine and heliotrine have been found to inhibit the swelling of isolated rat liver mitochondria in buffered sucrose solutions both in the presence or absence of added substrate. Inhibition of mitochondrial swelling indicated that the increased mitochondrial permeability to NAD caused by these alkaloids was due to a specific permeability change, and not to a general increase in mitochondrial permeability. N-Oxides of the pyrrolizidine alkaloids, lasiocarpine and heliotrine, did not influence the rate of mitochondrial swelling, indicating that the inhibitory effect of the alkaloids on swelling, like their effect on mitochondrial permeability to NAD, NAD status and metabolism, is associated with the positive charge on the ring nitrogen atom.

Iron and ruthenium stable complexes affected the swelling of mitochondria in sucrose solutions differently from the alkaloids. Iron complexes were found to accelerate swelling, and ruthenium complexes initially accelerated, then inhibited swelling. Acceleration of swelling by the complexes was largely prevented by the alkaloids or by ATP, but only to a small extent by sodium amytal.

Spontaneous swelling of mitochondria in KCl solutions was not influenced by the alkaloids or by the complexes.

During the experiments the mitochondrial suspensions were examined microscopically by phase-contrast to interpret correctly the observed optical density changes. The results of these examinations are given and their significance discussed.

INTRODUCTION

THE neuromuscular blocking agents, D-tubocurarine, pyrrolizidine alkaloids[1] and certain chemically stable inorganic complexes[2–6] have all been found to affect both neuromuscular impulse transmission and mitochondrial oxidative metabolism in a similar manner, related to the positive charge which they carry.[1, 7, 8] The effect of these agents on mitochondrial metabolism *in vitro* is to cause increased permeability of respiring mitochondria to NAD, the loss from mitochondria of oxidized NAD coenzymes and, consequently, respiratory failure due to inactivation of NAD-dependent enzyme systems.[7, 8] The increase in mitochondrial permeability to NAD

Abbreviations used in this paper: NAD, nicotinamide-adenine dinucleotide; ATP, adenosine triphosphate; EDTA, ethylenediamine-tetraacetic acid; $Ru(phen)_3^{++}$, tris-1; 1:10-phenanthroline ruthenium (II) perchlorate dihydrate; $Ru(trpy)_2^{++}$, bis-2:2′:2″-terpyridine ruthenium (II) perchlorate hemihydrate; $Fe(phen)_3^{++}$, tris-1:10-phenanthroline iron (II) perchlorate monohydrate; $Fe(bipy)_3^{++}$, tris-2:2′-bipyridine iron (II) perchlorate monohydrate.

leading to the loss of NAD coenzymes appeared to be a specific effect requiring active respiration by mitochondria in the presence of neuromuscular blocking agents, and the effect could be inhibited by sodium amytal which prevents the oxidation of reduced NAD.[7, 8] However the possibility remained that the neuromuscular blocking agents interfered with the mitochondrial membrane in a way, which led to a general nonspecific increase in mitochondrial permeability resulting in rapid mitochondrial swelling and leakage of NAD coenzymes. The present study was concerned with the investigation and evaluation of this possibility.

MATERIALS AND METHODS

Enzyme preparations. Rats were stunned and killed by decapitation .Livers were removed rapidly and immersed in ice-cold 0·25 M sucrose. Homogenates were prepared in 0·25 M sucrose and fractionated by differential centrifuging at 0–1°C.[9] Mitochondria were re-suspended in 0·25 M sucrose and re-isolated three times before use. Mitochondria were finally re-suspended in 0·25 M sucrose unless otherwise indicated in the text.

Reagents. Complex ions were obtained as gifts from the late Professor Dwyer of the Australian National University, Canberra. Pyrrolizidine alkaloids were obtained as gifts from Dr. Culvenor of the Chemical Research Laboratories, C.S.I.R.O. Other reagents used were the purest forms available commercially.

Methods. Mitochondrial swelling was assessed in the systems outlined in the text by the E_{520} value of mitochondrial suspensions, and verified by phase-contrast microscopy and photography.

RESULTS

Figures 1–3 show representative curves constructed from the mean results of at least four experiments each.

(a) *Effect of* D-*tubocurarine and pyrrolizidine alkaloids*

(i) *Effect on mitochondrial swelling in sucrose solutions.* Concentrations of D-tubocurarine or of the pyrrolizidine alkaloids, lasiocarpine and heliotrine, which cause increased permeability to NAD, the loss of NAD coenzymes and inactivation of NAD-dependent enzyme systems when incubated with respiring mitochondria,[7, 8] retarded considerably the swelling of liver mitochondria in buffered sucrose solutions. As illustrated in Fig. 1, for 1 mM D-tubocurarine, and in Fig. 2 for 16·7 mM lasiocarpine, the alkaloids stabilized mitochondria against swelling in 0·25 M sucrose as well as did 1·8 mM sodium amytal (Fig. 1), a recognized stabilizing agent.[10]

A similar protective effect of the alkaloids against mitochondrial swelling was obtained when either of the substrates, L-malate or succinate was added. This indicated that the stabilizing effect was not due to inhibition of respiration as succinate oxidation is not inhibited by the alkaloids.[7, 8] The alkaloids also inhibited the slower spontaneous swelling of mitochondria in 0·25 M sucrose which occurs in the absence of added substrate. Under these experimental conditions, when substrate is not added to the incubation mixture, mitochondrial respiration is supported solely by their endogenous substrates.[10]

(ii) *Effect of lasiocarpine and heliotrine N-oxides on mitochondrial swelling.* The N-oxides of lasiocarpine and heliotrine did not affect mitochondrial swelling, even when

concentrations ten-fold those of the parent alkaloid were used. Figure 2 shows that the rate of mitochondrial swelling in the presence of 16·7 mM lasiocarpine N-oxide was not significantly different from the rate of swelling of control mitochondria. As the N-oxides differ from the parent pyrrolizidine alkaloids in being uncharged, it seemed probable that the stabilizing effect of the alkaloids against mitochondrial swelling was associated with the charge on the ring nitrogen atom.

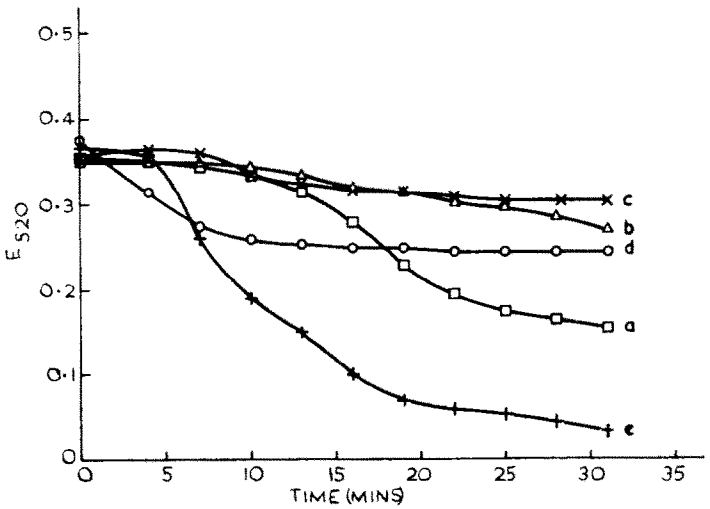

FIG. 1. Time change in E_{520} of mitochondrial suspensions incubated with: (a) no addition; (b) Na amytal 1·8 mM; (c) D-tubocurarine 1 mM; (d) 1-Ru $(phen)_3^{++}$ 0·2 mM; (e) DL-Fe $(phen)_3^{++}$ 0·2 mM. System: sucrose 0·25M; tris buffer, pH 7·4, 20 mM; L-malate 1 mM; thrice washed mitochondria equivalent to 100 mg fresh rat liver in 0·25M sucrose; water to 3·4 ml final volume; gas phase, air; temperature, 22°.

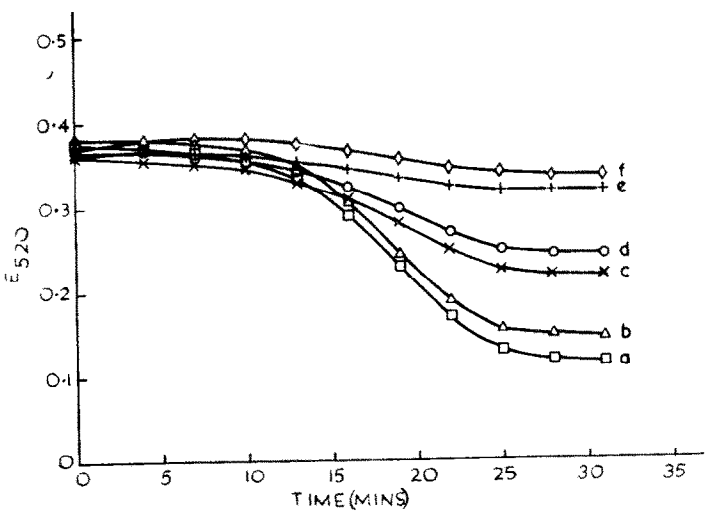

FIG. 2. Time change in E_{520} of mitochondrial suspensions incubated with: (a) no addition; (b) lasiocarpine 16·7 mM; (c) lasiocarpine N-oxide 16·7 mM; (d) DL-Fe $(phen)_3^{++}$ 10 μM; (e) DL-Fe $(phen_3^{++}$ 10 μM + D-tubocurarine 1 mM; (f) DL-Fe $(phen)_3^{++}$ 10 μM + Na amytal 1·8 mM. System: as for Fig. 1.

(b) *Effect of complex ions*

(i) *Effect of iron complexes on mitochondrial swelling in sucrose solutions.* Unlike the stabilizing effect of D-tubocurarine and the pyrrolizidine alkaloids, the addition of low concentrations, 10–200 μM, of either $Fe(phen)_3^{++}$ or $Fe(bipy)_3^{++}$ to mitochondrial suspensions in buffered 0·25 M sucrose caused a rapid fall in E_{520}. Phase-contrast microscopy confirmed that the fall in E_{520} was due to swelling of mitochondria. Figures 1 and 2 illustrate the decline in the E_{520} value of mitochondrial suspensions in the presence of 0·2 mM (Fig. 1) and 10 μM (Fig. 2) DL-$Fe(phen)_3^{++}$. The decline in E_{520} was more rapid with the higher concentration. Similar concentrations of $Fe(bipy)_3^{++}$ produced the same results as $Fe(phen)_3^{++}$. Mitochondrial swelling rates in the presence of concentrations lower than 10 μM of either $Fe(phen)_3^{++}$ or $Fe(bipy)_3^{++}$ did not differ significantly from control rates.

(ii) *Effect of ruthenium complexes on mitochondrial swelling in sucrose solutions.* The ruthenium complexes $Ru(phen)_3^{++}$ and $Ru(trpy)_2^{++}$ behaved in a manner intermediate between D-tubocurarine and the pyrrolizidine alkaloids on the one hand and and iron complexes on the other. Like the iron complexes, the ruthenium complexes initially accelerated swelling, as indicated by declining E_{520} values, but like D-tubocurarine and the pyrrolizidine alkaloids, the ruthenium complexes then stabilized the mitochondrial suspension at a much higher E_{520} value than that of the control suspension of mitochondria. This effect is illlustrated in Fig. 1, where the change in E_{520} in the presence of 0.2 mM 1-$Ru(phen)_3^{++}$ is plotted. Concentrations of 1-Ru-$(phen)_3^{++}$ from 0·1–0·6 mM produced almost the same degree and rate of change of E_{520} value. Higher concentrations than 0·6 mM caused aggregation and precipitation of mitochondria, and with lower concentrations than 0·1 mM the effects decreased until at 40 μM no significant deviation from control mitochondrial swelling was detectable.

Phase-contrast microscopy showed that the ruthenium complexes had two effects on mitochondria, some were swollen and some were dense and contracted.

The addition of either L-malate or succinate to mitochondria in sucrose solutions resulted in a quicker response both to iron and ruthenium complexes as compared with mitochondria in sucrose without added substrate.

(c) *Effect of* D-*tubocurarine, pyrrolizidine alkaloids and complex ions on mitochondrial swelling in KCl solutions*

None of the compounds tested was found to influence the rate of spontaneous mitochondrial swelling, as measured by E_{520} values, in media containing 0·15 M KCl instead of sucrose. Reversal of swelling by the addition of 2 mM ATP occurred as readily in the presence of D-tubocurarine, pyrrolizidine alkaloids, iron complexes or ruthenium complexes as with control suspensions of untreated mitochondria.

(d) *Prevention of mitochondrial swelling in the presence of complex ions*

The addition of 2 mM ATP to the 0·25 M sucrose suspending media containing either iron or ruthenium complexes completely prevented mitochondrial swelling as indicated by E_{520} measurements and confirmed by phase-contrast microscopy. However, once mitochondrial swelling was initiated by either iron or ruthenium complexes, the addition of 2 mM ATP had no influence on the subsequent change in E_{520} value.

Stabilization of mitochondria at approximately the initial E_{520} values was achieved

by the prior addition of 1 mM D-tubocurarine even in the presence of either iron or ruthenium complexes. This is illustrated in Fig. 2 by the curves showing E_{520} values of mitochondrial suspensions in the presence of 10 μM DL-Fe(phen)$_3^{++}$ with and without 1 mM D-tubocurarine. A similar degree of stabilization against 10 μM DL-Fe(phen)$_3^{++}$ was achieved by 16·7 mM lasiocarpine. However, when 16·7 mM lasiocarpine was added to reaction mixtures containing 50μM or more of iron or ruthenium complexes, it accelerated rates of decline in E_{520} values due to aggregation and sedimentation of mitochondria as shown by phase microscopy. Similar aggregation and sedimentation of mitochondria occurred when 16·7 mM lasiocarpine was added to mitochondria suspended in 0·25 M sucrose containing 20 μM or more $CaCl_2$. This also occurred when the concentrations of complexes were raised above 0·6 mM, and it may be a charge effect.

Although both D-tubocurarine and pyrrolizidine alkaloids as well as ATP prevented mitochondrial swelling due to Fe(phen)$_3^{++}$, sodium amytal had very little, if any effect. This is illustrated in Fig. 2, which shows stabilization of mitochondria by 1 mM D-tubocurarine against swelling due to 10 μM DL-Fe(phen)$_3^{++}$ but practically no protection by 1·8 mM sodium amytal.

(e) *Effect of the concentration of alkaloids and complexes on mitochondrial swelling.* It was considered that the different effects of the alkaloids and the complexes on mitochondrial swelling in sucrose solutions might have been due to differences in concentration, and that swelling might be inhibited by high concentrations of the complexes and stimulated by low concentrations of the alkaloids. Concentrations greater than 0·6 mM of the complexes aggregated mitochondria, so their effect on swelling could not be studied. However, it was found that lower concentrations of D-tubocurarine or pyrrolizidine alkaloids than required to inhibit swelling did not promote it. The concentration gradient effect for D-tubocurarine is illustrated in Fig. 3.

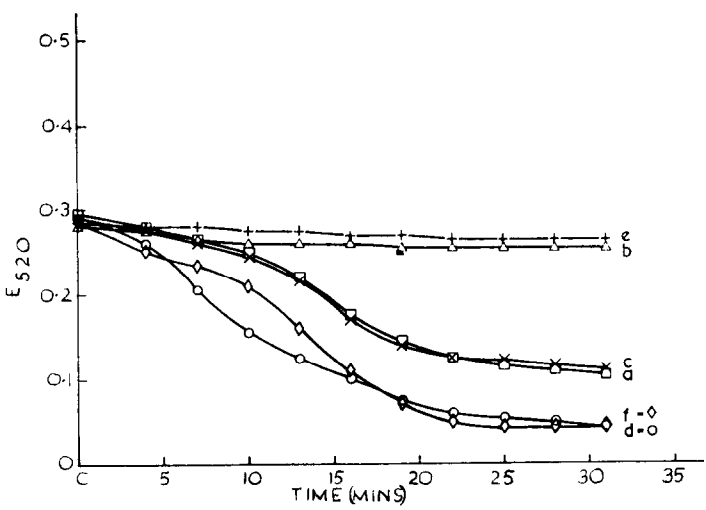

FIG. 3. Time change in E_{520} of mitochondrial suspensions incubated with: (a) no addition; (b) D-tubocurarine 0·1 mM; (c) D-tubocurarine 0·33 mM; (d) tubocurarine 0·67 mM; (e) D-tubocurarine 1 mM; (f) D-tubocurarine 2 mM. System: as for Fig. 1.

It can be seen in Fig. 3 that both 2 mM and 1 mM D-tubocurarine stabilized the mitochondrial suspensions against swelling. The higher concentration resulted in a more prolonged period of increased mitochondrial contraction and higher E_{520} value. Figure 3 shows that mitochondrial stability decreased with the d-tubocurarine concentration until at 0·1 mM the rate of decline in E_{520} value was little different from that for the control suspension of mitochondria. Lower D-tubocurarine concentrations than 0·1 mM did not alter the change in E_{520} value from the control rate.

(f) *Mitochondrial morphology*

Phase-contrast microscopy and photography were used to determine whether changes in the E_{520} value of mitochondrial suspensions were associated with changes in mitochondrial appearance.

Figure 4 shows control mitochondria 10 min after suspension in buffered 0·25 M sucrose containing 1 mM L-malate. The E_{520} value of the suspension had fallen only 0·015 and the mitochondra appeared as dense, dark spheres with only a few showing crescent formation indicative of the onset of swelling. However after 20 min incubattion the E_{520} of this mitochondrial suspension had fallen by 0·170 and, accordingly, Fig. 5 shows that many of the mitochondria were swollen, presenting "signet-ring" appearances due to pallor and crescent formation.

In agreement with the protection afforded by D-tubocurarine and pyrrolizidine alkaloids against swelling as indicated by E_{520} values, mitochondria were found to remain small, dense, dark spheres in the presence of these agents. This is illustrated in Fig. 6 which is a photograph of mitochondria 30 min after suspension in buffered 0·25 M sucrose containing 1 mM L-malate, 1 mM D-tubocurarine and 0·1 mM DL-Fe-$(phen)_3^{++}$. $Fe(phen)_3^{++}$ in the same system without D-tubocurarine caused the degree of mitochondrial swelling shown in Fig. 7, corresponding to a fall in E_{520} value from 0·300 to 0·051. The presence of 2 mM ATP in the suspending medium produced small, dense, dark mitochondria similar to those resulting from the addition of D-tubocurarine or pyrrolizidine alkaloids.

The behaviour of ruthenium complexes in causing an initial acceleration of mitochondrial swelling, as indicated by falling E_{520} values and then stabilization above the final E_{520} value of control suspensions of mitochondria, was reflected in the microscopic appearance of the mitochondria. Figure 8 shows mitochondria 30 min after suspension in buffered 0·25 M sucrose, 1 mM I-malate and 0·2 mM $Ru(phen)_3^{++}$. The E_{520} value of the suspension had fallen more rapidly for 7 min than that of a control suspension and had then remained stable about 0·100 above the final value for the control suspension. Figure 8 shows that some of the mitochondria had swollen, and some had become small, dense and dark.

DISCUSSION

Although d-tubocurarine and the pyrrolizidine alkaloids, heliotrine and lasiocarpine, caused increased permeability of mitochondria to NAD, and the loss from them of NAD coenzymes,[7, 8] the present study has shown that these alkaloids did not accelerate the swelling of mitochondria suspended in 0·25 M sucrose solution. On the contrary, both D-tubocurarine and the pyrrolizidine alkaloids stabilized mitochondria against swelling even in the presence of agents which promote swelling. The stabilizing effect, like the effect on mitochondrial permeability to NAD, NAD

status and respiratory enzyme systems, appeared to be associated with the positive charge carried by the ring nitrogen atom, as it was not produced by the same or ten-fold concentrations of the N-oxides of the pyrrolizidine alkaloids. As the alkaloids do not inhibit the oxidation of succinate but prevent mitochondrial swelling in the presence of this substrate, their stabilizing influence cannot be due simply to inhibition of respiration. However the protection against swelling may be associated with the inhibition of NAD dependent oxidation of endogenous substrates as in the experiments with the absence of added substrate.

The iron complexes on the other hand, accelerated mitochondrial swelling in 0·25 M sucrose, and the ruthenium complexes initially accelerated and then inhibited swelling. Unpublished studies in this laboratory have shown that both iron and ruthenium complexes initially accelerate oxidative metabolism by mitochondria, and ruthenium but not iron complexes then inhibit NAD-dependent enzyme systems. Therefore the initial accelerated rate of mitochondrial swelling in the presence of these agents may be due to increased mitochondrial respiration. Ultimate stabilization of the mitochondria against further swelling by ruthenium complexes may be due to the progressive inhibition of NAD-dependent enzyme systems; a property not displayed by iron complexes. In addition, iron complexes have been found to uncouple oxidative phosphorylation (unpublished data), and uncoupling of oxidative phosphorylation is itself a cause of mitochondrial swelling apart from that associated with electron transport.[10] Swelling of mitochondria in the presence of iron complexes is not prevented by amytal and is therefore not related to activity at the NAD section of the terminal respiratory chain. Prevention by ATP of swelling due to iron complexes indicates that the effect may in fact be associated with uncoupling of oxidative phosphorylation rather than acceleration of electron transport.

Phase-contrast microscopy and photography of mitochondrial suspensions during the course of the experiments was found to be essential for the correct interpretation of E_{520} changes in relation to mitochondrial swelling. For example, it was confirmed that the stable E_{520} values in the presence of D-tubocurarine of the pyrrolizidine alkaloids were due to the maintenance of mitochondria as small, dense spheres as distinct from the pale, "signet-ring", swollen mitochondria in suspensions with low E_{520} values. Use of the microscope enabled immediate recognition of the reason for rapid falls in E_{520} values in the presence of high concentrations of the complexes, or combinations of high concentrations of either the complexes or Ca^{++} and pyrrolizidine alkaloids as being due to clumping and sedimentation of mitochondria from suspension and not due to swelling. It is thought that the aggregation of mitochondria under these conditions may be due to a charge effect.

This study has provided further evidence of the similar effects of the alkaloids, D-tubocurarine, lasiocarpine and heliotrine on mitochondria, and that such effects are associated with the positive charge carried by the ring nitrogen. Although the alkaloids produce increased mitochondrial permeability to NAD, this change is specific and is not part of a general increase in mitochondrial permeability. A general increase in mitochondrial permeability would be associated with accelerated swelling in sucrose solutions whereas the alkaloids inhibited swelling. On the other hand a difference in the effects of the complexes on mitochondria from that of the alkaloids had become apparent. Iron complexes promoted mitochondrial swelling and ruthenium complexes

initially promoted and then inhibited swelling. The initial promotion of mitochondrial swelling by both iron and ruthenium complexes may be due to acceleration of respiration, and the prolonged effect of iron complexes to uncoupling of oxidative phosphorylation, properties which do not appear to be shared by D-tubocurarine or the pyrrolizidine alkaloids.

Acknowledgements—We wish to thank Mr. D. S. Roberts, M.V.Sc. and Mr. I. Roper for photography, and Misses Olga Korukina, Lana Korukina and Kay Holland for careful technical assistance.

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