Biochemical Pharmacology, Vol. 17, pp. 533–538. Pergamon Press. 1968. Printed in Great Britain

THE EFFECTS OF NEUROMUSCULAR BLOCKING AGENTS ON MITOCHONDRIA—IV.

EFFECTS OF *d*-TUBOCURARINE, PYRROLIZIDINE ALKALOIDS AND MAGNESIUM ON OXIDATIVE PHOSPHORYLATION

C. H. GALLAGHER

Department of Veterinary Pathology, Faculty of Veterinary Science, University of Sydney, Australia

(*Received* 7 *June* 1967; *accepted* 27 *October* 1967)

Abstract—The effects of *d*-tubocurarine and the pyrrolizidine alkaloids, lasiocarpine and heliotrine on oxidative phosphorylation and oxidations by liver mitochondria were studied under conditions supplying inadequate and adequate Mg.

Oxidative phosphorylation in Mg-deficient systems was considerably depressed by 10 μM *d*-tubocurarine, 100 μM lasiocarpine and 1 mM heliotrine, concentrations lower than required to inhibit NAD-dependent oxidations. No effect on the efficiency phosphorylation was observed at any concentration of the alkaloids in the presence of 6·7 mM $MgSO_4$. Oxidative phosphorylation accompanying succinate oxidation was not significantly affected in systems containing either sufficient or deficient Mg.

Addition of Mn or Co did not relieve the inhibition of oxidative phosphorylation imposed by the alkaloids in Mg-deficient systems.

Concentrations of the alkaloids which depressed oxidative phosphorylation under Mg-deficient conditions did not activate or stimulate the breakdown of ATP.

Oxidations of NAD-dependent substrates by mitochondria were found to be more susceptible to inhibition by the alkaloids under Mg-deficient conditions.

The uncharged *N*-oxides of lasiocarpine and heliotrine had no effect on oxidative phosphorylation or on oxidations by mitochondria in the presence or absence of adequate Mg.

The effect of the alkaloids on oxidative phosphorylation and oxidations under Mg-deficient conditions was not due to induction, acceleration or increase of the loss of Mg from mitochondria. Mitochondrial Mg levels remained stable during oxidations in 6·7 mM $MgSO_4$ and fell at the same rate during incubation without added Mg whether alkaloids were present or not.

The results are discussed in relation to the hypothesis that *d*-tubocurarine and the pyrrolizidine alkaloids compete as cations with NAD for sites of attachment within mitochondria. The presence of adequate Mg is antagonistic to the effects of the alkaloids on NAD-dependent metabolism.

EARLIER studies[1-3] showed that alkaloids of the pyrrolizidine group and *d*-tubocurarine inhibit both neuromuscular transmission and mitochondrial oxidative metabolism by virtue of the cationic charge on the ring nitrogen atoms. Although the alkaloids facilitate access of NAD to enzyme systems in intact mitochondria under *in vitro* conditions[2, 3] and cause the loss of NAD co-enzymes from mitochondria both *in vitro*[2, 3] and *in vivo*,[4] they inhibit the swelling of mitochondria suspended in buffered

Abbreviations used in this paper: ATP, adenosine triphosphate; NAD, nicotinamide-adenine dinucleotide; AMP, adenosine-5-monophosphate; EDTA, ethylenediamine-tetraacetic acid.

sucrose solutions[5] and influence cationic fluxes of isolated mitochondria in specific and different ways which preclude the possibility of a general increase in mitochondrial permeability.[6] Consequently an hypothesis that the alkaloids, acting as similarly charged cations, competitively displace NAD from sites of attachment within mitochondria is being investigated.

The observation was made in these and other studies that reversal of inhibition of oxidations by NAD-dependent enzyme systems of mitochondria is not accompanied by restoration of normal oxidative phosphorylation efficiency. Presumably added NAD gains access to sites appropriate for electron transport much more readily than to the precise loci required for oxidative phosphorylation. Therefore, the possibility was considered that agents displacing NAD from attachment sites in mitochondria would disconnect phosphorylation from electron transport before causing such a dislocation of NAD as to inhibit oxidations. The present study was designed to evaluate this possibility.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing 150–200 g were used.

Mitochondrial preparations. Rats were stunned and killed by decapitation. Livers were removed rapidly and immersed in ice-cold 0·25 M sucrose. Homogenates in 0·25 M sucrose were prepared in a Dounce-type hand homogeniser and fractionated by differential centrifuging at 0–1°.[7] Mitochondria were resuspended in 0·25 M sucrose and re-isolated three times before use.

Methods. Oxygen uptake was measured by Warburg manometers. Inorganic phosphate was measured by the method of Fiske and Subba Row.[8]

The standard system used for assaying oxidative phosphorylation was: AMP 1 mM; $MgSO_4$ 6·7 mM; KCl 25 mM; Na-K inorganic orthophosphate buffer, pH 7·4, 13·3 mM; cytochrome *c* 10 μM; glucose 30 mM; hexokinase 300 units; L-glutamate 10 mM; NaF 10 mM; mitochondria equivalent to 100 mg fresh liver in 0·5 ml 0·25 M sucrose; final volume 3 ml; temperature 38°; gas phase air; equilibration period 10 min.

Magnesium was measured by atomic absorption spectrophotometry using a Techtron instrument as described by Willis.[9–11]

RESULTS

(a) *Effect on oxidative phosphorylation in a complete system*

Table 1 shows a typical experiment in which, under the usual conditions of measuring oxidative phosphorylation, P/O ratios for L-glutamate oxidation were not altered by 1 mM *d*-tubocurarine, 3·3 mM lasiocarpine or 33 mM heliotrine, although oxygen uptake was strongly inhibited. Concentrations of 0·5 mM *d*-tubocurarine, 1·67 mM lasiocarpine and 6·7 mM heliotrine slightly inhibited oxidation but did not alter P/O ratios. Lower concentrations of the alkaloids affected neither oxidation nor phosphorylation.

(b) *Effect on oxidative phosphorylation in a Mg-deficient system*

Observations made during the study of alkaloid influences on cationic fluxes[6] indicated the possibility that *d*-tubocurarine, the pyrrolizidine alkaloids and Mg competed as cations in certain respects. Consequently the effect of the alkaloids on oxidative phosphorylation in the absence of added Mg was investigated.

When Mg was omitted from the system used for oxidative phosphorylation, both *d*-tubocurarine and lasiocarpine depressed P/O ratios (Table 2). The absence of Mg in control experiments lowered P/O ratios considerably for glutamate oxidation but only slightly for succinate oxidation. However, the addition of 10 μM *d*-tubocurarine or 100 μM lasiocarpine still further lowered the P/O ratios for glutamate oxidation

TABLE 1. EFFECT OF ALKALOIDS ON OXIDATIVE PHOSPHORYLATION

Additions	Oxygen uptake (μl/30 min)	P/O (μg atoms P esterified/μg atom O used)
None	113	2·4
d-Tubocurarine 1 mM	52	2·2
Lasiocarpine 3·3 mM	56	2·4
Heliotrine 33 mM	41	2·5

System: as in 'Methods'.

TABLE 2. EFFECT OF ALKALOIDS ON OXIDATIVE PHOSPHORYLATION IN AN MG-DEFICIENT SYSTEM

Substrate	Mg addition	P/O (μg atoms P esterified/μg atom O used)		
		Control	*d*-Tubocurarine 10 μM	Lasiocarpine 100 μM
L-Glutamate 10 mM	Mg 6·7 mM	2·5 ± 0·3	2·5 ± 0·3	2·4 ± 0·3
L-Glutamate 10 mM	No Mg	1·6 ± 0·3	0·7 ± 0·2	0·9 ± 0·2
Succinate 10 mM	Mg 6·7 mM	1·5 ± 0·3	1·4 ± 0·2	1·6 ± 0·3
Succinate 10 mM	No Mg	1·3 ± 0·3	1·1 ± 0·3	1·4 ± 0·3

System: as for Table 1 with the omission of $MgSO_4$. Results are the means of 5 experiments.

to about half the control value in the absence of added Mg. Oxidations of both L-glutamate and succinate proceeded at normal rates in the absence of added Mg with or without 10 μM *d*-tubocurarine or 100 μM lasiocarpine. These concentrations of the alkaloids had no effect on P/O ratios in the presence of 6·7 mM $MgSO_4$ and had no effect on oxygen uptake by mitochondria oxidizing glutamate or succinate in the presence or absence of added Mg. The addition of 3·3 mM $MgSO_4$ or of 13·3 mM $MgSO_4$ had the same effect as 6·7 mM $MgSO_4$ in preventing the depression of phosphorylation encountered under Mg-deficient conditions and in countering the augmented inhibition of phosphorylation induced by 10 μM *d*-tubocurarine or 100 μM lasiocarpine under such conditions. Table 1 shows a slight but insignificant depression of P/O ratio when *d*-tubocurarine was added to mitochondria oxidizing succinate without the addition of Mg. As AMP was used as the phosphate acceptor in these experiments and requires myokinase activity for oxidative phosphorylation to proceed, the possibility arose that lower P/O ratios in Mg-deficient systems might have been associated with inactivation of the Mg-dependent enzyme, myokinase. In order to evaluate this possibility, experiments were carried out using ADP as the phosphate acceptor instead of AMP. The results were the same with either acceptor thus excluding the participation of myokinase as a relevant factor.

Higher concentrations of 0·5 mM–2 mM *d*-tubocurarine and 0·5 mM–10 mM lasiocarpine inhibited the oxidation of glutamate by mitochondria both with and without added Mg but did not alter P/O ratios from the appropriate control levels.

Heliotrine had a similar but less powerful effect to lasiocarpine, requiring 10-fold concentrations.

Lasiocarpine-*N*-oxide and heliotrine-*N*-oxide at, above and below the concentrations of lasiocarpine and heliotrine which were tested, had no effect on oxidative phosphorylation with or without added Mg.

Neither Mn nor Co tested over a wide range of concentrations raised the P/O ratio for glutamate oxidation in a Mg-deficient system with or without the alkaloids.

(c) *Effect of* EDTA

It was considered possible that EDTA could simulate a Mg-deficient system by chelating Mg and thus render mitochondria susceptible to interference by low concentrations of the alkaloids. However, experiments showed that 0·1–2 mM EDTA had no effect on oxidative phosphorylation with or without the alkaloids.

(d) *Effect of* d-*tubocurarine and lasiocarpine on* ATP-*ase*

The alkaloids did not stimulate ATP-ase activity in mitochondria metabolizing in the absence of added Mg.

(e) *Effect of* d-*tubocurarine and lasiocarpine on mitochondrial Mg*

Acceleration or exaggeration of the loss of Mg from mitochondria suspended in a medium deficient in Mg was considered as a possible mechanism by which the alkaloids could influence oxidative phosphorylation. This possibility was evaluated in a series of experiments employing atomic absorption spectrophotometry to measure mitochondrial Mg.

Experiments were terminated by diluting reaction mixtures 15-fold with ice-cold 0·25 M sucrose followed immediately by centrifuging at 0–2° at 27,000 *g* for 3 min to isolate mitochondria. The tubes were drained and dried carefully and the mitochondrial pellet resuspended in a 'swamping solution' containing 15,000 ppm $SrCl_2$, 10,000 ppm NaCl and 100,000 ppm KCl for Mg analysis.

TABLE 3. EFFECT OF ALKALOIDS ON MITOCHONDRIAL MG

Mg addition	Incubation time (min)	Mitochondrial Mg (μM/g dry mitochondria)					
		Control		*d*-Tubocurarine 10 μM		Lasiocarpine 100 μM	
		Range	Mean	Range	Mean	Range	Mean
6·7 mM	0	186–206	196	190–194	192	178–205	191
6·7 mM	30	195–222	208	180–200	190	188–205	196
Nil	0	29– 37	33	31– 35	33	31– 37	34
Nil	10	13– 19	16	14– 22	18	17– 30	24
Nil	20	13– 16	15	14– 32	23	17– 30	24
Nil	30	6– 18	11	9– 20	13	4– 16	11

System: as for Table 2. Results are the means of 2 experiments in duplicate.

Table 3 shows that neither 10 μM *d*-tubocurarine nor 100 μM lasiocarpine caused the loss of Mg from mitochondria performing oxidative phosphorylation with or without added Mg.

These experiments showed that mitochondria metabolizing in the presence of added 6·7 mM Mg had high initial levels of Mg, 178–206 μM/g dry mitochondria, and maintained these values during incubation at 38°. On the other hand, mitochondria metabolizing in the absence of added Mg had low initial concentrations of Mg, 29–37 μM/g dry mitochondria, which fell progressively with incubation at 38°. However, the presence of the alkaloids did not accelerate the rate of loss of mitochondrial Mg.

(f) *Effect of alkaloids and Mg on oxidations*

Further evidence of the influence of Mg on the susceptibility of mitochondria to the effects of the alkaloids was obtained by studying oxidative enzyme systems.

Mitochondria oxidizing NAD-dependent substrates without added Mg were more rapidly and more severely inactivated than when Mg was added. This effect was seen with both L-glutamate and L-malate as substrates, and over a range of concentrations of 0·5–2 mM *d*-tubocurarine and 1–6·7 mM lasiocarpine. Table 4 illustrates the greater

TABLE 4. EFFECT OF ALKALOIDS AND MG ON L-MALATE OXIDATION

Mg addition	Time (min)	Interval O uptake				
		Control	*d*-Tubocurarine 0·5 mM		Lasiocarpine 1 mM	
		μl	μl	%Inhibition	μl	%Inhibition
Nil	20	87	70	20	51	41
	40	63	42	33	42	33
	60	58	21	64	40	31
	Total	208	133	36	133	36
$MgSO_4$	20	87	83	5	79	9
6·7 mM	40	75	72	5	75	0
	60	68	60	12	72	0
	Total	230	215	6	226	2

System: as for Table 1 with the omission of $MgSO_4$, glucose, hexokinase and NaF, and the replacement of L-glutamate by 10 mM L-malate.

susceptibility of Mg-deficient mitochondria to inhibition of L-malate oxidation by the lower concentrations of the alkaloids. In the presence of 6·7 mM Mg, 0·5 mM *d*-tubocurarine or 1 mM lasiocarpine did not inhibit L-malate oxidation but were rapidly inhibitory in the absence of added Mg.

The alkaloids did not inhibit succinate oxidation with or without Mg.

DISCUSSION

These experiments have shown that mitochondria are much more susceptible to the effects of the alkaloids *d*-tubocurarine and lasiocarpine when the reaction mixture is deficient in Mg. In the absence of added Mg, concentrations of either alkaloid lower than required for inhibition of mitochondrial oxidation systems, considerably depress the esterification of phosphate during oxidative phosphorylation by NAD-dependent enzyme systems. The alkaloids do not depress P/O ratios in the presence of 6·7 mM Mg, even when added in concentrations high enough to inhibit oxidations. The uncharged derivative of lasiocarpine, lasiocarpine-*N*-oxide, does not influence oxidative phosphorylation with or without Mg.

The effect of the alkaloids in a Mg-deficient system is not due to acceleration of, or exaggeration of the loss of Mg from mitochondria. It is conceivable that the alkaloids displace Mg from sites within mitochondria appropriate for oxidative phosphorylation but this seems unlikely in view of the inability of the alkaloids to depress significantly oxidative phosphorylation accompanying succinate oxidation by mitochondria without added Mg.

The results favour the hypothesis that *d*-tubocurarine and lasiocarpine compete with NAD as cations for sites within metabolizing mitochondria to produce initially a sufficient dislocation of NAD from precise loci to block the associated phosphorylation step without interrupting electron transport. Further displacement of NAD leads to disruption of electron transport. Both the effect of the alkaloids on oxidative phosphorylation and NAD-dependent oxidations are facilitated by incubation of mitochondria in a Mg-deficient medium. Mg, too, may compete with the alkaloids as a cation or it may protect NAD against the effects of the alkaloids.

Acknowledgements—I wish to thank Mrs. V. Reeve for skilled research assistance throughout this study.

REFERENCES

1. C. H. GALLAGHER and J. H. KOCH, *Nature, Lond.* **183**, 1124 (1959).
2. C. H. GALLAGHER, *Biochem. Pharmac.* **3**, 220 (1960).
3. J. H. KOCH and C. H. GALLAGHER, *Biochem. Pharmac.* **3**, 231 (1960).
4. G. S. CHRISTIE and R. N. LE PAGE, *Biochem. J.* **84**, 25 (1962).
5. J. H. KOCH and C. H. GALLAGHER, *Biochem. Pharmac.* **14**, 237 (1965).
6. C. H. GALLAGHER and J. D. JUDAH, *Biochem. Pharmac.* **16**, 883 (1967).
7. W. C. SCHNEIDER, *J. biol. Chem.* **176**, 259 (1948).
8. C. H. FISKE and Y. SUBBA ROW, *J. biol. Chem.* **66**, 375 (1925).
9. J. B. WILLIS, *Spectrochim. Acta* **16**, 259 (1960).
10. J. B. WILLIS, *Analyt. Chem.* **34**, 614 (1962).
11. J. B. WILLIS, *Proc. R. Aust. Chem. Inst.* 245 (1962).