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THE EFFECTS OF NEUROMUSCULAR BLOCKING AGENTS ON MITOCHONDRIA—III

EFFECTS OF D-TUBOCURARINE AND PYRROLIZIDINE ALKALOIDS ON THE UPTAKE AND DEPLETION OF CATIONS

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Abstract—The effects of D-tubocurarine and the pyrrolizidine alkolaids, lasiocarpine and heliotrine, on mitochondrial fluxes of K^+, Na^+, Mg^{2+} and Ca^{2+} were studied and compared with DNP,* which increases mitochondrial permeability, and nupercaine which stabilizes mitochondrial permeability.

Unlike DNP, the alkaloids did not cause immediate H^+-uptake by fresh mitochondria.

The pyrrolizidine alkaloids decreased K^+-loss from mitochondria incubated in K^+-free media, whereas D-tubocurarine increased it. K^+-loss caused by D-tubocurarine was largely prevented by lasiocarpine and, to a lesser extent, by nupercaine. However, the protective effect of lasiocarpine was completely eliminated by DNP.

H^+-evolution upon adding KCl to K^+-depleted mitochondria was inhibited by both D-tubocurarine and the pyrrolizidine alkaloids. Correspondingly, the K^+-uptake was inhibited. Inhibition of the H^+-out/K^+-in exchange was not due only to inhibition of substrate oxidation, because it was shown by washed mitochondria without added substrate as well as during succinate oxidation, which the alkaloids do not inhibit.

Neither D-tubocurarine nor the pyrrolizidine alkaloids influenced the loss of Na^+, Mg^{2+} or Ca^{2+} from mitochondria, or the uptake of Na^+. The uptake of Mg^{2+} and Ca^{2+} by mitochondria was inhibited by both D-tubocurarine and the pyrrolizidine alkaloids when oxidizing substrates by NAD-dependent enzyme systems.

It was concluded that D-tubocurarine and the pyrrolizidine alkaloids, lasiocarpine and heliotrine, do not cause a general increase in mitochondrial permeability but produce their effects on mitochondria by more specific mechanisms related to the positive charge carried on the ring N. The hypothesis is proposed that the alkaloids compete with other positively charged compounds for sites in mitochondria and thus actively displace NAD from such sites.

INTRODUCTION

IN EARLIER studies[1–3] it was found that alkaloids of the pyrrolizidine group and D-tubocurarine inhibited both neuromuscular transmission and mitochondrial oxidative metabolism by virtue of the positive charge on the ring nitrogen atoms. Although these alkaloids were found to facilitate access of NAD to enzyme systems

* Abbreviations used: NAD, nicotinamideadenine dinucleotide; ATP, adenosine triphosphate; EDTA, ethylenediamine-tetraacetic acid; Tris, 2-amino–2-hydroxymethylpropane–1: 3-diol; TCA, trichloroacetic acid; DNP, 2, 4-dinitrophenol.

in intact mitochondria under *in vitro* conditions[2, 3] and to cause the loss of NAD coenzymes from mitochondria under both *in vitro*[2, 3] and *in vivo*[4] conditions, it was found subsequently that they inhibited the swelling of isolated mitochondria suspended in buffered sucrose solutions. This indicated that facilitation by the alkaloids of the movement of NAD into and out of mitochondria is a specific effect rather than being due to a general increase in mitochondrial permeability.

The present study was designed to investigate further the effect of D-tubocuarine and pyrrolizidine alkaloids on mitochondrial permeability by studying the influence of the alkaloids on the movement of mono- and di-valent inorganic cations into and out of mitochondria.

MATERIALS AND METHODS

Animals, mitochondrial preparations, dry weight, cation and phosphate determinations, H^+-uptake and -efflux measurements were measured as described in earlier papers.[6–8]

RESULTS

a. *Effect on H^+-uptake by fresh mitochondria*

Fresh rat-liver mitochondria suspended in 0·25M sucrose containing 0·1–1mM EDTA at pH 6·3–7·2 do not take up H^+ unless acted upon by certain reagents.[7] For example, the addition of 20 μM DNP, which increases mitochondrial permeability

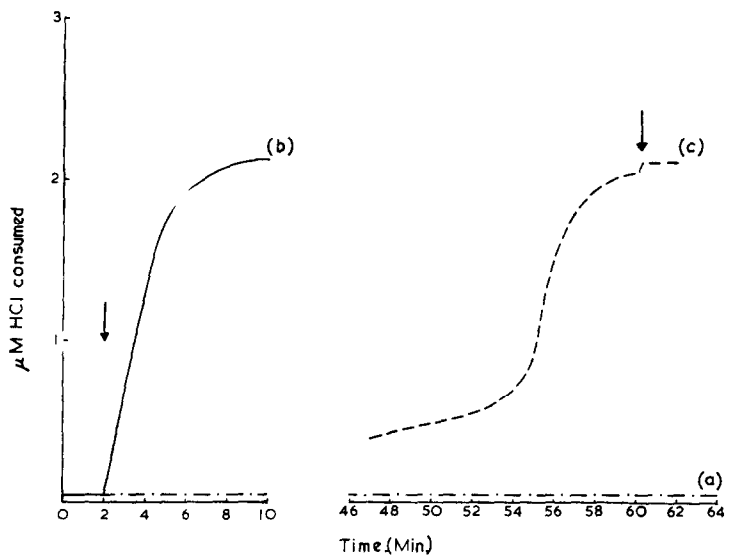

FIG. 1. H^+-uptake by fresh mitochondria.

System: Rat-liver mitochondria (25 mg dry wt) prepared in 0·25M sucrose; sucrose 0·2M; EDTA 0·1mM; pH 6·7; final volume 5·0 ml; 20°. Reagents were adjusted to pH 6·7 prior to use and any subsequent change in pH corrected quickly. H^+-uptake was measured by titration to an end-point of pH 6·7 by means of an automatic titrator and recorder used in conjunction with an automatic micro-burette containing 0·2N HCl.

Curve (a) = H^+-uptake by control mitochondria. Curve (b) = H^+-uptake by mitochondria with 20μM DNP added at the 1st arrow. Curve (c) = H^+-uptake by mitochondria with 1mM d-tubocurarine added at the 1st arrow and with 20μM DNP added at the 2nd arrow.

to cations, causes a rapid uptake of H^+ (Fig. 1). The mitochondria become saturated with H^+ and exhibit no further uptake when more DNP is added.

Short-term experiments indicated that 1mM D-tubocurarine did not cause H^+-uptake by mitochondria. However, when the experiments were prolonged a slow rate of H^+-uptake by mitochondria occurred in the presence of D-tubocurarine after about 45–50 min at pH 7·0, about 25–30 min at pH 6·7, and about 5 min at pH 6·3. The rate increased slowly and then terminated in a rapid burst of H^+-uptake similar to that shown by mitochondria incubated with DNP (Fig. 1). The mitochondria did not then exhibit any further uptake of H^+ upon addition of either DNP or D-tubocurarine.

Mitochondria incubated with 1–10mM lasiocarpine or heliotrine, or the N-oxide of either pyrrolizidine alkaloid, did not take up H^+ or lose responsiveness to DNP over a period of 70 min at pH 6·3–7·0. However, the experiments were complicated by continuing slow consumption of HCl by solutions of the pyrrolizidine alkaloids in this pH range in the absence of mitochondria, probably due to the formation of HCl derivatives of traces of the alkaloids present as free base.

b. *Effect on H^+-efflux from K^+-depleted mitochondria*

Mitochondria which have been depleted of K^+ accumulate H^+.[6] Such mitochondria

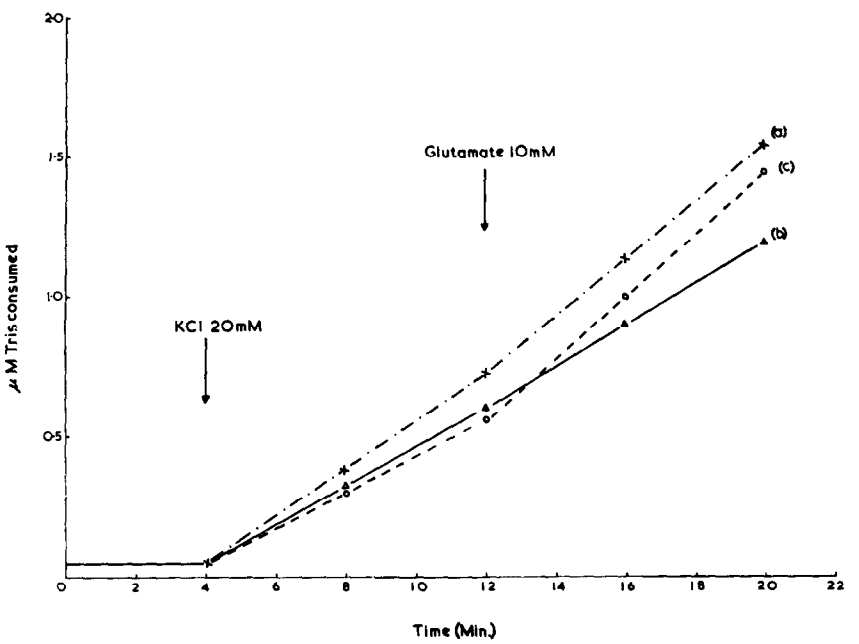

FIG. 2. H^+-efflux by K^+-depleted mitochondria.

System: Rat-liver mitochondria (15 mg dry wt) suspended in 2·6 ml 0·25M sucrose/1mM EDTA were incubated at 20° for 30 min to deplete K^+ and then transferred quantitatively to beakers by means of a 1 ml 0·25M sucrose/1mM EDTA wash. The pH was adjusted to 7·2 and corrected quickly after adding reagents. Final volume was 4 ml, 20°. H^+-efflux was measured by titration to PH 7·2 with an automatic titrator, recorder and burette containing 0·01 M Tris, pH 10·4 H^+-efflux was initiated by adding 20 mM KCl at the 1st arrow and accelerated by addidg 10 mM L-glutamate at the 2nd arrow. Curve (a) = H^+-efflux by control mitochondria. Curve (b) = H^+-efflux by mitochondria with 1 mM D-tubocvrarine added at zero time. Curve (c) = H^+-efflux by mitochondria with 5 mM lasiocarpine added at zero time.

will then exchange H^+ for K^+ when the latter are presented to them especially when oxidizing a substrate.[6]

Figure 2 shows that the addition of either 1mM D-tubocurarine or 5mM lasiocarpine retarded the rate of H^+-evolution from K^+-depleted mitochondria upon adding KCl. Subsequent addition of L-glutamate had little effect on the rate of H^+-efflux in the presence of D-tubocurarine but accelerated the rate of both the control and lasiocarpine systems (Fig. 2). The lack of response to the addition of L-glutamate to the reaction mixture containing D-tubocurarine was probably due to strong inhibition of the oxidation of L-glutamate by 1mM D-tubocurarine.

When mitochondria were depleted of K^+ in the presence of 1mM D-tubocurarine or 5mM lasiocarpine, the efflux of H^+ upon addition of KCl was found to be considerably inhibited (Fig. 3). Even greater inhibition was produced by depletion of mitochondria in the presence of 10mM lasiocarpine. Figure 3 also shows that the addition of L-glutamate produced a similar effect of the alkaloids on H^+-efflux from K^+-depleted mitochondria to that shown in Fig. 2, but the differences in rate were greater in this system where mitochondria were depleted in the presence of the alkaloids than in the prior system where mitochondria were depleted before adding the alkaloids. Mitochondria depleted with 1mM D-tubocurarine showed an accelerated rate of H^+-efflux upon addition of L-glutamate in the presence of KCl but this rate

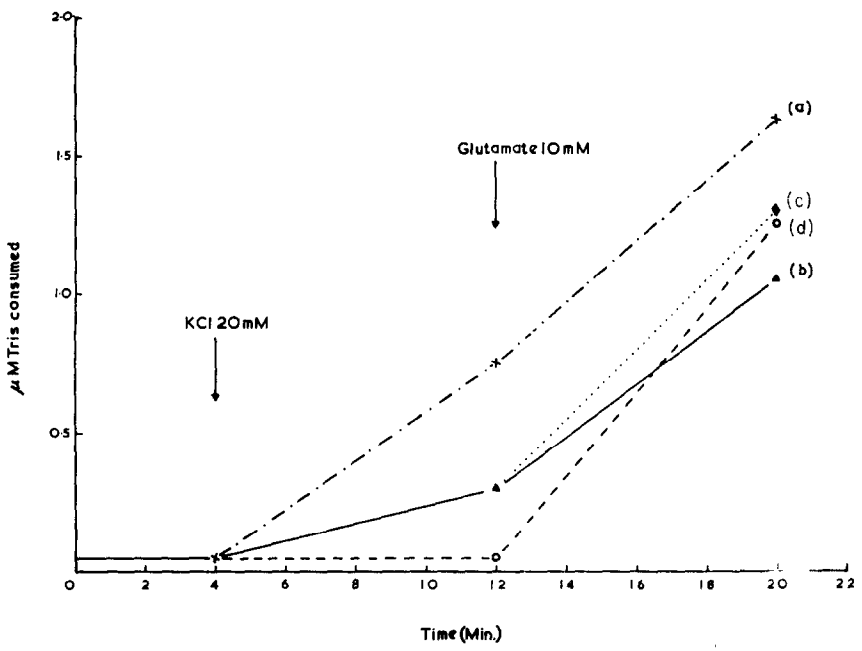

FIG. 3. H^+-efflux by mitochondria depleted of K^+ in the presence of alkaloids.

System: Rat-liver mitochandria (15 mg dry wt) were depleted of K^+ as for Fig. 2 but with alkaloids added before depletion. Other conditions for the measurement of H^+-efflux are as in Fig. 2.

Curve (a) = H^+-efflux by control mitochondria.

Curve (b) = H^+-efflux by mitochondria depleted in the presence of 1 mM D-tubocurarine.

Curve (c) = H^+-efflux by mitochondria depleted in the presence of 5 mM lasiocarpine.

Curve (d) = H^+-efflux by mitochondria depleted in the presence of 10 mM lasiocarpine.

TABLE 1. K^+ OF MITOCHONDRIA IN EXPERIMENTS COMPRISING FIGS. 2 AND 3

Additions	K^+ (mEquiv/kg dry mitochondria)													
	Fig. 2							Fig. 3						
		Fresh	K^+-depleted		Incubated with K^+		K^+-uptake during incubation	Fresh	K^+-depleted		Incubated with K^+			K^+-uptake during incubation
	Range	Mean	Range	Mean	Range	Mean	Mean	Range	Mean	Range	Mean	Range	Mean	Mean
None	146–156	151	70–95	80	133–147	142	62	140–151	147	42–49	45	123–139	130	85
D-Tubocurarine 1mM	146–156	151	70–95	80	95–106	103	23	140–151	147	32–37	35	98–112	107	72
Lasiocarpine 5mM	146–156	151	70–95	80	124–133	127	47	140–151	147	45–59	51	120–125	123	72
Lasiocarpine-N-oxide 10mM	146–156	151	70–95	80	135–138	136	56	140–151	147	42–53	46	130–133	132	86

System: As in Figs. 2 and 3. Mitochondria were isolated for K^+-analysis from one of each pair of flasks immediately upon commencement of titration of its duplicate. Results are the means of three experiments.

was less than that shown by control mitochondria, which, in turn, was less than that shown by mitochondria depleted in the presence of 5 or 10mM lasiocarpine.

Heliotrine influenced H^+-efflux from K^+-depleted mitochondria in the same manner as did lasiocarpine.

The N-oxide of neither lasiocarpine nor heliotrine had any effect on H^+-efflux from K^+-depleted mitochondria.

The concentrations of K^+ in mitochondria used in the experiments comprising Figs. 2 and 3 are recorded in Table 1. The values agree quite well with those indicated by the titrigraph.

c. *Effect on K^+-efflux from mitochondria*

Determination of K^+ concentrations in experiments on H^+ movement (Table 1, Fig. 3) indicated an effect of the alkaloids on the loss of K^+ from mitochondria. The presence of 1mM D-tubocurarine during incubation of mitochondria to deplete them of K^+ appeared to increase K^+-loss, whereas the presence of 5mM lasiocarpine appeared to decrease the loss. Lasiocarpine-N-oxide did not appear to have an influence. The results of experiments designed to investigate effects of the alkaloids on K^+-depletion are summarized in Table 2.

TABLE 2. EFFECT OF ALKALOIDS ON K^+-EFFLUX FROM MITOCHONDRIA

Additions	K^+ (mEquiv/kg dry mitochondria)				
	Fresh		K^+-depleted		K^+-loss
	Range	Mean	Range	Mean	
None	130–137	135	65–72	68	67
D-Tubocurarine 1mM	130–137	135	36–46	43	92
Lasiocarpine 5mM	130–137	135	80–89	85	50
Lasiocarpine-N-oxide 10mM	130–137	135	69–74	72	63

System: Mitochondria (about 15 mg dry wt/flask) were incubated with constant shaking for 20 min at 38° in 0·2M sucrose, 50mM Tris HCl, pH 7·4, and 1mM EDTA. Results are the means of four experiments.

Table 2 shows that 1mM D-tubocurarine considerably increased the loss of K^+ from mitochondria under conditions appropriate for K^+-depletion, 5mM lasiocarpine decreased the loss of K^+ and 10mM lasiocarpine-N-oxide had no significant effect. This pattern remained constant under different conditions of depletion involving alteration of the incubation temperature from 38° to 20°, addition of 10mM L-glutamate or 10mM succinate, addition of 20mM KCl, reduction of the sucrose concentration from 0·2M to 50mM and omission of EDTA.

Heliotrine at 10mM had about the same effect as 5mM lasiocarpine. Heliotrine-N-oxide had no effect.

d. *Effect on K^+-uptake by K^+-depleted mitochondria*

The presence of 1mM D-tubocurarine considerably retarded the uptake of K^+ by K^+-depleted mitochondria when incubated with 20mM KCl under conditions appropriate to K^+-uptake (Table 3). Inhibition of K^+-uptake by 1mM D-tubocurarine

TABLE 3. EFFECT OF ALKALOIDS ON K^+-UPTAKE BY K^+-DEPLETED MITOCHONDRIA

Alkaloid	Substrate	K^+ (mEquiv/kg dry mitochondria)											
		Alkaloids added before K^+-depletion						Alkaloids added after K^+-depletion					
		Fresh		K^+-loss during depletion		K^+-uptake during 10 min incubation in 20mM KCl		Fresh		K^+-loss during depletion		K^+-uptake during 10 min incubation in 20mM KCl	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
None	L-Glutamate	119–140	129	52–60	56	35–47	43	127–142	134	49–59	54	43–58	49
D-Tubocurarine 1mM	L-Glutamate	119–140	129	74–83	77	5–10	8	127–142	134	49–59	54	8–20	17
Lasiocarpine 5mM	L-Glutamate	119–140	129	46–53	51	28–31	30	127–142	134	49–59	54	33–41	38
Lasiocarpine-N-oxide 10mM	L-Glutamate	119–140	129	54–63	60	40–56	51	127–142	134	49–59	54	35–49	42
None	Succinate	125–141	133	58–70	65	55–64	61	—	—	—	—	—	—
D-Tubocurarine 1mM	Succinate	125–141	133	85–97	88	12–27	18	—	—	—	—	—	—
Lasiocarpine 5mM	Succinate	125–141	133	46–55	52	46–58	53	—	—	—	—	—	—

System: Mitochondria (about 15 mg dry wt/flask) were depleted of K^+ by incubation for 20 min at 38° in 0·2M sucrose, 50mM Tris HCl, pH 7·4, and 1 mM EDTA. K^+-uptake was measured after incubation for 10 min at 38° in 0·2M sucrose, 50mM Tris HCl, pH 7·4, 20mM KCl, 1mM inorganic orthosphosphate, pH 7·4, and 10mM substrate. Results are the means of four experiments.

occurred with either L-glutamate or succinate as substrate, showing that the effect is not simply due to inhibition of L-glutamate oxidation as D-tubocurarine does not inhibit succinate oxidation.[3] D-Tubocurarine depressed K^+-uptake whether added before or after the initial incubation of mitochondria to deplete them of K^+.

Table 3 also shows that 5mM lasiocarpine, which would inhibit L-glutamate oxidation but not succinate oxidation,[2] depressed the uptake of K^+ by K^+-depleted mitochondria regardless of whether the substrate was L-glutamate or succinate, and whether the lasiocarpine was added to mitochondria before or after incubation for depletion. Lasiocarpine N-oxide at 10mM did not depress K^+-uptake by mitochondria when added before incubation for K^+-depletion but depressed K^+-uptake slightly when added after K^+-depletion.

Heliotrine, 10mM, had an effect similar to that of 5mM lasiocarpine, and 10mM heliotrine-N-oxide had no effect.

e. *Factors affecting alkaloid influences on the loss and uptake of K^+ by mitochondria*

Judah *et al.*[7] demonstrated that DNP accelerates the loss of K^+ from mitochondria during incubation for K^+-depletion, and inhibits the subsequent uptake of K^+ by depleted mitochondria when they are incubated under appropriate conditions. The same authors found that the local anaesthetic, nupercaine, which stabilizes the semipermeability of biological membranes generally, protects mitochondria against the effects of DNP on K^+ flux. It was of interest, therefore, to determine (a) whether nupercaine protected mitochondrial K^+ against the effects of D-tubocurarine and (b) whether lasiocarpine protected against the effects of DNP in accelerating K^+-loss and inhibiting K^+-uptake.

TABLE 4. EFFECT OF ALKALOIDS, DNP AND NUPERCAINE ON THE LOSS AND UPTAKE OF K^+ BY MITOCHONDRIA

Additions	K^+ (mEquiv/kg dry mitochondria)		
	Fresh	K^+-loss during depletion	K^+-uptake during incubation with KCl
None	121	38	32
D-Tubocurarine 2mM	121	85	32
D-Tubocurarine 2mM + nupercaine 0·1mM	121	72	26
D-Tubocurarine 2mM + lasiocarpine 10mM	121	48	20
Lasiocarpine 10mM	121	33	27
Lasiocarpine 10mM + DNP 20μm	121	92	18
DNP 20μm	121	66	−14
DNP 20μm + nupercaine 0·1mM	121	50	—
Nupercaine 0·1mM	121	37	29

System: Mitochondria (about 19mg dry wt/flask) were depleted of K^+ by incubation for 20 min at 20° in 0·2M sucrose, 50mM Tris HCl, pH 7·4, 1mM EDTA and other reagents as indicated in Table 4. K^+-uptake was measured after incubation for 40 min at 20° in 20mM KCl, 10mM L-glutamate, 1mM inorganic orthophosphate, pH 7·4 and 50mM Tris HCl, pH 7·4. Results are the means of two experiments.

The data summarized in Table 4 show that 0·1M nupercaine afforded some protection (28 per cent) against the loss of K^+ from mitochondria caused by 2mM D-tubocurarine but did not influence K^+-uptake. The same concentration of nupercaine protected against 57 per cent of the loss of K^+ induced by 20 μM DNP.

Lasiocarpine, 10mM, afforded good protection (79 per cent) against the loss of K^+ caused by 2mM D-tubocurarine but did not influence K^+-uptake (Table 4). Surprisingly, lasiocarpine did not give any protection against the effect of DNP on K^+-loss but augmented it considerably. Lasiocarpine did, however, cause an uptake of K^+ by mitochondria in the presence of 20 μM DNP, which when alone produced further loss of K^+ under conditions designed to facilitate the uptake of K^+ from 20 mM KCl.

The counter-effects of D-tubocurarine and lasiocarpine on the loss of K^+ from mitochondria were further investigated by varying the concentrations of each alkaloid, as shown in Table 5. It was found that 10mM lasiocarpine almost completely protected mitochondria against the loss of K^+ induced by 2mM D-tubocurarine. Similar protection was afforded by 1mM lasiocarpine against 0·4mM D-tubocurarine. Less, but still considerable, protection against K^+-loss from mitochondria during incubation with 2mM D-tubocurarine was given by 1mM lasiocarpine.

TABLE 5. COUNTER-EFFECTS OF D-TUBOCURARINE AND LASIOCARPINE ON THE LOSS OF K^+ FROM MITOCHONDRIA

Additions	K^+ (mEquiv/kg dry mitochondria)	
	Fresh	K^+-loss during depletion
None	154	53
D-Tubocurarine 2mM	154	102
D-Tubocurarine 0·4mM	154	91
Lasiocarpine 10mM	154	46
Lasiocarpine 1mM	154	57
D-Tubocurarine 2mM + lasiocarpine 10mM	154	60
D-Tubocurarine 2mM + lasiocarpine 1mM	154	73
D-Tubocurarine 0·4mM + lasiocarpine 1mM	154	62

System: Mitochondria (about 15 mg dry wt/flask) were depleted of K^+ by incubation for 15 min at 20° in 0·2M sucrose, 50mM Tris HCl, pH 7·4 and 1mM EDTA. Results are the means of two experiments.

f. *Effect on the Na^+-status of mitochondria*

Fresh mitochondria do not contain much Na^+ but lose a small amount when incubated in buffered sucrose/EDTA. In the present experiments, the Na^+ concentrations fell from 39 to 34 mEquiv./kg dry mitochondria during 20 min incubation at 38°. The presence of D-tubocurarine, lasiocarpine or lasiocarpine-N-oxide during incubation appeared to protect against such loss but this was found to be due to the addition of Na^+ with the alkaloids, and was eliminated when EDTA salts were used.

g. *Effect on the loss and uptake of Mg^{2+} by mitochondria*

Incubation of mitochondria in buffered sucrose/EDTA solutions in the absence of exogenous substrate or Mg^{2+} leads to the loss of Mg^{2+} as shown in Table 6. Addition of 1mM D-tubocurarine, 5mM lasiocarpine or 10mM lasiocarpine-N-oxide did not influence the loss of Mg^{2+}.

Mitochondria accumulate Mg^{2+} when incubated in a buffered medium containing Mg^{2+} and inorganic phosphate, especially when a substrate is also present (Table 6). The uptake of Mg^{2+} in the absence of added substrate, and in the presence of 10mM succinate was not significantly affected by 1mM D-tubocurarine, 5mM lasiocarpine

TABLE 6. EFFECT OF ALKALOIDS ON THE LOSS AND UPTAKE OF Mg^{2+} BY MITOCHONDRIA

Substrate	Mg^{2+} (mM/kg dry mitochondria)											
	Control				D-Tubocurarine 1mM			Lasiocarpine 5mM			Lasiocarpine-N-oxide 10 mM		
	Fresh	Depleted	Incubated with Mg^{2+}		Depleted	Incubated with Mg^{2+}		Depleted	Incubated with Mg^{2+}		Depleted	Incubated with Mg^{2+}	
			20°	38°		20°	38°		20°	38°		20°	38°
None	31	23	50	52	23	50	42	23	47	44	24	49	52
L-Glutamate 10mM	31	23	67	114	23	47	50	23	48	57	24	68	110
Succinate 10mM	31	23	72	149	23	66	154	23	72	154	24	70	152

System: Mitochondria (about 15 mg dry wt/flask) were depleted by incubating for 20 min at 38° in 0·22M sucrose, 50mM Tris HCl, pH 7·4m 1mM EDTA and alkaloids as indicated or an equal volume of water. Mg^{2+}-uptakewas initiated by adding 0·5 ml of a stock to give 10mM $MgSO_4$, 2mM inorganic orthophosphate, pH 7·4, 50mM Tris HCl, pH 7·4 and 10mM substrate in 5 ml final volume. The flasks were then incubated for 10 min at 20° or 38° as indicated. Mitochondria were re-isolated for Mg^{2+}-analysis as in Methods. Results are the means of two experiments.

TABLE 7. EFFECT OF ALKALOIDS ON THE LOSS AND UPTAKE OF Ca^{2+} BY MITOCHONDRIA

Substrate	Ca^{2+} (mM/kg dry mitochondria)								
	Control			D-Tubocurarine 1mM		Lasiocarpine 5mM		Lasiocarpine-N-oxide 10mM	
	Fresh	Depleted	Incubated with Ca^{2+}	Depleted	Incubated with Ca^{2+}	Depleted	Incubated with Ca^{2+}	Depleted	Incubated with Ca^{2+}
None	14	6	26	7	14	7	14	7	34
L-Glutamate 10mM	14	6	57	7	30	7	45	7	62
Succinate 10mM	14	6	59	7	53	7	62	7	60

System: Mitochondria (about 15 mg dry wt/flask) were depleted as for Table 6. Ca^{2+}-uptake was initiated by adding 0·5 ml of a stock to give 0·2 mM $CaCl_2$, 50mM Tris HCl, pH 7·4 and 10mM substrate in 5 ml final volume. The flasks were then incubated for 3 min at 20°. Mitochondria were re-isolated for Ca^{2+}-analysis as in Methods. Results are the means of two experiments.

or 10mM lasiocarpine-N-oxide. However, both 1mM D-tubocurarine and 5mM lasiocarpine completely inhibited the uptake of Mg^{2+} associated with the oxidation of L-glutamate. Either of these alkaloids at the concentration used inhibits the oxidation by mitochondria of L-glutamate but not of succinate. Lasiocarpine-N-oxide, which is not inhibitory to mitochondrial oxidations, did not affect Mg^{2+}-uptake by mitochondria during L-glutamate oxidation, Table 6 also shows that although the uptake of Mg^{2+} during 10 min was not altered by incubation at either 20° or 38° in the absence of an added substrate, it was about doubled by incubation at the higher temperature when a substrate was added.

An effect equivalent to that of 5mM lasiocarpine was produced by 10mM heliotrine.

h, *Effect on the loss and uptake of Ca^{2+} by mitochondria*

Normal mitochondria from liver do not contain much Ca^{2+}, about 14mM/kg dry mitochondria. Incubation in buffered sucrose/EDTA solution without added substrate causes them to lose about half of their Ca^{2+}, as shown in Table 7. The addition of Ca^{2+} to the incubation medium results in the uptake of Ca^{2+} by mitochondria, especially when a substrate is added. The conditions of incubation are critical for the study of such an uptake of Ca^{2+}, as a point is reached either by prolonged incubation at 20° or by incubation at 38° at which the Ca^{2+} concentration disrupts mitochondrial metabolism and causes the loss of Ca^{2+} and other cations. However, it was ascertained that incubation for 3 min at 20° in 0·2M $CaCl_2$ did not damage mitochondria detectably and produced repeatable results.

Table 7 shows that neither the alkaloids, D-tubocurarine and lasiocarpine, nor lasiocarpine-N-oxide influenced the loss of Ca^{2+} from mitochondria during depletion conditions. However, although 10mM lasiocarpine-N-oxide did not affect the subsequent uptake of Ca^{2+} initiated by adding $CaCl_2$ with or without a substrate, both 1mM D-tubocurarine and 5mM lasiocarpine did. The uptake of Ca^{2+} by mitochondria in the absence of added substrate was depressed by both alkaloids, and the augmented Ca^{2+}-uptake resulting from adding 10mM L-glutamate was also reduced, especially by D-tubocurarine. However, the degree of inhibition by the alkaloids of Ca^{2+}-uptake during glutamate oxidation by mitochondria was not so great as for the uptake of Mg^{2+} under conditions appropriate for Mg^{2+}-uptake. Neither 1mM D-tubocurarine nor 5mM lasiocarpine affected Ca^{2+}-uptake by mitochondria during the oxidation of succinate.

Heliotrine at 10mM had approximately the effect of 5mM lasiocarpine, and 10mM heliotrine-N-oxide had no effect.

DISCUSSION

These studies have shown that the effect of D-tubocurarine and of the pyrrolizidine alklaoids, lasiocarpine and heliotrine, in causing an apparent increase in the permeability of mitochondria to the loss and uptake of NAD co-enzymes,[1–4] is not simply a consequence of a general increase in permeability of mitochondrial membranes. This conclusion is in accordance with the observation that D-tubocurarine and the pyrrolizidine alkaloids protect mitochondria against imbibition of water and swelling when suspended in buffered sucrose solutions.[5]

In addition, unlike their similar effects on mitochondria with regard to oxidation systems, NAD status and propensity to swelling, specific differences, in fact, antagon-

isms, have been demonstrated in the effects of D-tubocurarine and the pyrrolizidine alkaloids on the movement of H^+ and K^+ into and out of mitochondria.

Nupercaine and DNP are known to influence antagonistically the permeability of mitochondria to ionic movement, nupercaine protecting against the increase in permeability otherwise caused by DNP.[7] The effects of these reagents were therefore used as criteria in determining the possible disturbances in mitochondrial permeability to ions induced by D-tubocurarine and the pyrrolizidine alkaloids.

Unlike DNP, neither D-tubocurarine nor the pyrrolizidine alkaloids caused an immediate uptake of H^+ by fresh mitochondria. The pyrrolizidine alkaloids had no effect but D-tubocurarine caused a delayed onset of H^+-uptake which culminated in a burst of activity to the extent that the mitochondria were no longer capable of further H^+-uptake when DNP was added. The effects of the alkaloids on K^+-efflux from fresh mitochondria in K^+-free media corresponded to their influences on H^+-influx. D-Tubocurarine increased K^+-loss, whereas the pyrrolizidine alkaloids decreased the loss of K^+ below control values.

Even allowing for the different conditions of incubation, there appears to be a discrepancy in time, but not in degree, between H^+-influx and K^+-efflux in the presence of D-tubocurarine, whereas DNP causes an immediate burst of H^+-uptake corresponding to a similar K^+-loss. An explanation of this discrepancy may be in the earlier observation that mitochondria take up D-tubocurarine.[3] The loss of K^+ from mitochondria caused by D-tubocurarine may be compensated initially by the uptake of ionized D-tubocurarine, and subsequently by the uptake of H^+ as the D-tubocurarine is metabolized or converted to an un-ionized form.

When mitochondria depleted of K^+ by prior incubation in K^+-free sucrose/EDTA, were incubated under conditions appropriate for the uptake of K^+ and the corresponding evolution of H^+, it was found that either D-tubocurarine or the pyrrolizidine alkaloids inhibited both K^+-influx and H^+-efflux. The uptake of K^+, and thus indirectly the exchange of H^+ for K^+, by mitochondria is an endergenic process requiring the oxidation of a substrate for optimal activity. Inhibition of substrate oxidation could thus play a rôle in preventing K^+-uptake and H^+-loss and is unquestionably of some importance in retarding the $K^+ : H^+$ exchange associated with glutamate oxidation, which is inhibited by both D-tubocurarine and the pyrrolizidine alkaloids.[2, 3] However, the alkaloids do not inhibit succinate oxidation by mitochondria, yet reduce the uptake of K^+ when this substrate is being oxidized, showing that the effect is not due solely to oxidative inhibition.

Increased K^+-loss from fresh mitochondria in the presence of D-tubocurarine was antagonized strongly by lasiocarpine and, to a lesser extent, by nupercaine. However, the apparent stabilizing effect of lasiocarpine was of no avail against the loss of mitochondrial K^+ induced by DNP although countering DNP sufficiently to permit a small uptake of K^+ by K^+-depleted mitochondria under appropriate conditions.

The alkaloids did not influence the loss of Na^+, Mg^{2+} or Ca^{2+} from fresh mitochondria, nor the uptake of Na^+. However, both D-tubocurarine and the pyrrolizidine alkaloids affected the uptake of Mg^{2+} and Ca^{2+} by mitochondria.

Mitochondrial uptake of Mg^{2+} without added substrate or with succinate was not affected by the alkaloids, but the uptake associated with L-glutamate oxidation was completely inhibited by D-tubocurarine and lasiocarpine. The uptake of Ca^{2+} by mitochondria was similarly affected. The alkaloids inhibited Ca^{2+} uptake driven by

L-glutamate oxidation but not by succinate oxidation. The results indicate that the effects of D-tubocurarine and the pyrrolizidine alkaloids on the mitochondrial uptake of Mg^{2+} and Ca^{2+} are largely due to the ability of the alkaloids to inhibit NAD-dependent oxidations and not to specific effects on ionic movement.

As with earlier studies[1-3, 5] on other parameters, the effects on mitochondria of the pyrrolizidine alkaloids, lasiocarpine and heliotrine, differed only in degree, lasiocarpine being effective at lower concentrations than heliotrine. Again as in the earlier studies, the effects of the alkaloids appear to be associated with the cationic charge carried by the ring N, as the N-oxide of either pyrrolizidine alkaloid was without influence on the uptake or loss of cations by mitochondria.

The effects of D-tubocurarine and the pyrrolizidine alkaloids on mitochondrial metabolism involving NAD co-enzymes is clearly not due simply to the loss of NAD from mitochondria as a result of a general increase in mitochondrial permeability, but to more specific effects. Active displacement of NAD from a site of attachment within the mitochondrion by competition for the site by positively-charged compounds provides a working hypothesis to explain the mechanism of action of the alkaloids in inhibiting mitochondrial metabolism.[2, 3]

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. G. S. CHRISTIE, K. AHMED, A. E. M. MCLEAN and J. D. JUDAH, *Biochim. Biophys Acta* **94**, 432 (1965).
7. J. D. JUDAH, A. E. M. MCLEAN, K. AHMED and G. S. CHRISTIE, *Biochim. Biophys Acta* **94**, 441 (1965).
8. J. D. JUDAH, K. AHMED, A. E. M. MCLEAN and G. S. CHRISTIE, *Biochim. Biophys. Acta* **94**, 452 (1965).