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THE EFFECTS OF NEUROMUSCULAR BLOCKING AGENTS ON MITOCHONDRIA—I

EFFECTS OF *d*-TUBOCURARINE AND OF COMPLEX IONS ON MITOCHONDRIAL ENZYME SYSTEMS*

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Abstract—*d*-Tubocurarine and some complex ions, e.g. tris-1 : 10-phenanthroline ruthenium-II perchlorate dihydrate, inhibit oxidations by liver mitochondrial enzyme systems which require pyridine nucleotides for electron transfer, and stimulate succinate oxidation by mitochondria in 0·25 M sucrose. The compounds do not affect the activity of cytochrome oxidase or the efficiency of oxidative phosphorylation. Neither *d*-tubocurarine nor 1-Ru $(phen)_3^{2+}$ inhibit L-malic dehydrogenase directly, nor does *d*-tubocurarine stimulate succinate oxidation by mitochondria in water. However, 1-Ru $(phen)_3^{2+}$ inhibits the succinoxidase system of water-disrupted mitochondria.

d-Tubocurarine and 1-Ru $(phen)_3^{2+}$ influence oxidations by mitochondria in 0·25 M sucrose by increasing mitochondrial permeability and accelerating the loss of pyridine nucleotides. Inhibition of L-malate oxidation is largely prevented by adding excess DPN, and slightly prevented by EDTA. Mitochondria take up *d*-tubocurarine during incubation and some, at least, of the alkaloid becomes localized in the insoluble, membraneous fraction.

Active oxidation is necessary both for the development of inhibition by *d*-tubocurarine and for mitochondrial uptake of the alkaloid. The uptake of *d*-tubocurarine and of added DPN by mitochondria oxidizing succinate is prevented by Na amytal. Both increased permeability and *d*-tubocurarine uptake appear to be associated with the re-oxidation of reduced pyridine nucleotides. Similar effects of *d*-tubocurarine, pyrrolizidine alkaloids and complex ions on liver mitochondria and on neuromuscular junctions suggest that the compounds may act similarly at either tissue site and that such action may be referable to the charge carried.

INTRODUCTION

IN AN earlier study,[1] pyrrolizidine alkaloids were found to inhibit impulse transmission across neuromuscular junctions in a manner which resembled in some respects the neuromuscular blocking action of *d*-tubocurarine. Pyrrolizidine alkaloids have been shown also to inhibit the activity of certain enzyme systems of liver mitochondria.[2] The inhibitory locus of the pyrrolizidine alkaloids operative for either neuromuscular or mitochondrial systems appears to be the nitrogen atom of the cyclic base. Oxidation at the ring nitrogen to form N-oxides removes the inhibitory

* Abbreviations used in this paper: AMP, adenosine-5-monophosphate; DPN, diphosphopyridine nucleotide; GSH, reduced glutathione; EDTA, ethylenediaminetetraacetic acid; Ru $(phen)_3^{2+}$, tris-1 : 10-phenanthroline ruthenium-II perchlorate dihydrate; Ru $(trpy)_2^{2+}$, bis-2 : ′ : 2″-terpyridine ruthenium-II perchlorate hemihydrate.

quality of the pyrrolizidine alkaloids for either system. As *d*-tubocurarine blocks the transmission of impulses at neuromuscular junctions by virtue of its two quaternary nitrogen atoms, it was of interest to determine whether this alkaloid also influenced the activity of mitochondrial enzyme systems.

During the study it became pertinent to investigate the possible effects on mitochondrial enzymes of the chemically stable complex ions,[3] which exert a curare-like effect at neuromuscular junctions by nature of the charge they carry.[4–7] A preliminary account of some of this study has been published.[8]

MATERIALS AND METHODS

Enzyme preparations. Rats were killed by stunning and 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 °.[9] Mitochondria were resuspended in 0·25 M sucrose unless otherwise indicated. Hexokinase was prepared from yeast by a modification[10] of the method of Berger *et al.*[11] The fraction precipitating between 25 per cent and 40 per cent by volume of ethanol was dissolved in 1 per cent glucose solution and stored frozen. The optimal addition for oxidative phosphorylation was determined by assay.

Reagents. Complex ions were obtained as gifts for earlier studies from Dr. Dwyer of the National University, Canberra. AMP, DPN, nicotinamide, GSH, cytochrome *c*, citrate, α-oxoglutarate, L-glutamate, succinate, L-malate, oxaloacetate, octanoate, L-ascorbic acid, EDTA, *d*-tubocurarine, ethanol, sucrose and inorganic reagents were obtained commercially.

Succinic acid was purified by recrystallizing three times from hot water: then dissolved in 1N NaOH and precipitated as sodium succinate with ethanol. Sodium succinate was redissolved and crystallized from 80 per cent ethanol. Octanoic acid was purified by distillation *in vacuo*. L-Ascorbic acid and α-oxoglutaric acid were dissolved and neutralized with NaOH just before use. Other substrates and AMP were neutralized and stored frozen as sodium salts for 3–4 weeks.

Methods. O_2 uptake was measured by Warburg manometers. Spectrophotometric measurements were made with a Beckman model DU instrument. *d*-Tubocurarine was measured by the method of Pride and Smith,[12] and inorganic phosphorus by the method of Fiske and Subba Row.[13]

RESULTS

All experiments were done in duplicate and the results expressed are the means of at least repeated experiments.

(a) *Effect on oxidative metabolism*

(i) *Effect of* d-*tubocurarine on mitochondrial oxidations.* Table 1 shows that 1 mM *d*-tubocurarine inhibited the oxidation by mitochondria of citrate, α-oxoglutarate, L-malate, L-glutamate and octanoate but stimulated the oxidation of succinate. Lower concentrations of *d*-tubocurarine had much less effect on mitochondrial metabolism; 0·5 mM was hardly inhibitory to L-malate oxidation and 0·1 mM not at all so. The inhibitory effect of *d*-tubocurarine was slight during the first 10 min of each experiment but developed progressively to severe inhibition within 30 to 40 min. Experiments in which the alkaloid was added from side-arm compartments of Warburg

flasks during the course of oxdative experiments, demonstrated well the progressive development of inhibition.

TABLE 1. EFFECT OF *d*-TUBOCURARINE ON OXIDATIONS BY LIVER MITOCHONDRIA

Additions	Oxygen uptake (μl/30 min)				
	Control	*d*-Tubocurarine (1 mM)	*l*-Ru (phen)$_3^{2+}$ (0·5 mM)	*d*-Ru (phen)$_3^{2+}$ (0·5 mM)	Ru (trpy)$_2^{2+}$ (0·5 mM)
Citrate 10 mM	95	44	—	—	—
α-Oxoglutarate 10 mM	112	63	—	—	—
Succinate 10 mM	165	222	238	—	—
L-Malate 10 mM	108	55	55	62	73
L-Glutamate 10 mM	108	52	—	—	—
Octanoate 1·67 mM + L-malate 0·67 mM	129	69	—	—	—

System: AMP 1 mM; $MgSO_4$ 6·7 mM; KCl 25 mM; phosphate buffer, pH 7·4, 13·3 mM; cytochrome *c* 10 μM; mitochondria equivalent to 100 mg of fresh rat liver were added in 0·5 ml of 0·25 M sucrose except for succinate oxidation where mitochondria equivalent to 50 mg of fresh liver were added in 0·5 ml of 0·25 M sucrose; water to 3·0 ml final volume; 0·1 ml of 20 per cent KOH was placed in the centre well to absorb CO_2; gas phase, air; temperature, 38°; equlibration period 10 min.

d-Tubocurarine was found to affect oxidative metabolism by liver homogenates in 0·25 M sucrose in a similar manner to its effect on isolated mitochondria.

(ii) *Effect of complex ions on mitochondrial oxidations.* The complex ions, *l*- and *d*-Ru (phen)$_3^{2+}$ and Ru (trpy)$_2^{2+}$ inhibited L-malate oxidation by liver mitochondria (Table 1). Also *l*-Ru (phen)$_3^{2+}$, the only complex ion tested for such activity, was found to stimulate succinate oxidation as did *d*-tubocurarine (Table 1). Like *d*-tubocurarine, Ru (phen)$_3^{2+}$ became progressively inhibitory to L-malate oxidation.

(iii) *Effect of* d-*tubocurarine and* l-*Ru (phen)*$_3^{2+}$ *on oxidative phosphorylation.* Neither 1 mM *d*-tubocurarine nor 0·5 mM *l*-Ru (phen)$_3^{2+}$ lowered the efficiency of oxidative phosphorylation by liver mitochondria oxidizing L-malate, although oxidation was reduced by either compound.

(iv) *Effect of* d-*tubocurarine and* l-*Ru (phen)*$_3^{2+}$ *on cytochrome oxidase activity.* The cytochrome oxidase activity of rat liver homogenates in water was assayed by the method of Schneider and Potter[14] in the presence of 1 mM *d*-tubocurarine or of 0·5 mM *l*-Ru (phen)$_3^{2+}$. Neither compound had an effect on cytochrome oxidase.

(v) *Effect of* d-*tubocurarine and* l-*Ru (phen)*$_3^{2+}$ *on* L-*malic dehydrogenase.* The total activity of L-malic dehydrogenase was assayed in aqueous homogenates of rat liver in order to determine whether *d*-tubocurarine or *l*-Ru (phen)$_3^{2+}$ had a direct effect on the enzyme. Homogenates were prepared in water and allowed to stand at 0–1° for 45 min to ensure that osmotic rupture of mitochondria had occurred. L-Malic dehydrogenase activity was assayed by the method of Potter.[15] In this method the reaction mixture is supplemented with DPN to substitute for DPN lost from mitochondria due to water-cracking, and with L-glutamate to remove oxaloacetate by transamination to aspartate.

Neither 1 mM *d*-tubocurarine nor 0·5 mM *l*-Ru (phen)$_3^{2+}$ had any effect on L-malic dehydrogenase activity when assayed in this system.

(b) *Reversal of* d-*tubocurarine and of* l-*Ru (phen)*$_3^{2+}$ *inhibition*

(i) *Effect of respiratory cofactors.* Inhibition of mitochondrial enzyme systems requiring pyridine nucleotides for electron transfer and not of the succinoxidase system or of L-malic dehydrogenase directly in the presence of excess DPN indicated that the loss or inactivation of mitochondrial pyridine nucleotides might be involved. Incubation of 0·5 mM DPN, 0·67 mM GSH and 40 mM nicotinamide with the reaction mixture before adding *d*-tubocurarine or *l*-Ru (phen)$_3^{2+}$ largely prevented the development of inhibition of L-malate oxidation by either compound. Further, the addition of such a cofactor supplement at the same time as *d*-tubocurarine or *l*-Ru (phen)$_3^{2+}$ was also efficient in preventing inhibition of L-malate oxidation as shown in Table 2. The important cofactor in protecting against the inhibitory effects was found to be DPN, which gave good protection alone.

(ii) *Effect of EDTA.* A lesser degree of protection against inhibition of L-malate oxidation by *d*-tubocurarine or by *l*-Ru (phen)$_3^{2+}$ was obtained by the addition of 1 mM EDTA as shown in Table 2. Higher concentrations of EDTA did not give better protection. Inhibition by either compound was still severe when mitochondria were isolated from livers homogenized in 0·25 M sucrose containing 1 mM EDTA and resuspended in either 0·25 M sucrose or 0·25 M sucrose + 1 mM EDTA. The rate of development of inhibition was slightly retarded with mitochondria prepared from sucrose—EDTA homogenates as compared with sucrose homogenates, and further slightly retarded by the inclusion of 1 mM EDTA in the Warburg flasks.

TABLE 2. EFFECT OF RESPIRATORY COFACTORS AND EDTA ON INHIBITION OF L-MALATE OXIDATION

Additions	Oxygen uptake (μl/40 min)		
	Control	*d*-Tubocurarine (1 mM)	*l*-Ru (phen)$_3^{2+}$ (0·5 mM)
None	159	42	37
Cofactors	170	141	153
EDTA 1 mM	164	99	76
EDTA 1 mM + cofactors	187	164	159

System: As in Table 1 + L-malate 10 mM. Cofactor supplement: DPN 0·5 mM, GSH 0·67 mM and nicotinamide 40 mM.

The effect of EDTA on *d*-tubocurarine inhibition might possibly be due to altering the Ca : Mg ratio of mitochondria by chelation of Ca^{2+}. However, increased addition of Mg^{2+} had no effect on the rate of development or degree of inhibition of L-malate oxidation by the alkaloid.

Slightly better protection against inhibition by *d*-tubocurarine or by *l*-Ru (phen)$_3^{2+}$ was obtained consistently by the inclusion of EDTA as well as cofactors in the reaction mixture but the difference as compared with cofactors alone was always small (cf. Table 2).

(c) *Mechanism of inhibition by* d-*tubocurarine and by* l-*Ru(phen)*$_3^{2+}$

(i) *Effect of substrate.* Mitochondria were incubated for 20 min with 1 mM *d*-tubocurarine in the respiratory system shown in Table 3 without the addition of a substrate. No inhibition of endogenous oxidation was observed, but the rate was very low in any case. L-Malate was then added from side-arm compartments to the reaction mixture. Inhibition of oxidation was not evident for 20–30 min after the addition of L-malate and then developed progressively (as shown in Table 3 under "no substrate"). This showed that the inhibitory effect of *d*-tubocurarine was not due to a direct effect on mitochondria but was dependent upon oxidation by the mitochondria of an added substrate.

Further evidence on the necessity of oxidation of a substrate for *d*-tubocurarine inhibition to be expressed was obtained by studying mitochondria isolated from liver homogenates prepared in 0·25 M sucrose containing 1 mM *d*-tubocurarine. The mitochondria were washed by suspension in 0·25 M sucrose, re-isolated and resuspended in 0·25 M sucrose. Oxidation of L-malate by such mitochondria was normal and the mitochondria were not abnormally susceptible to inhibition by *d*-tubocurarine added to the Warburg flasks.

L-Malate was added to the main compartments of Warburg flasks after pre-incubation of mitochondria with *d*-tubocurarine in the presence of 0·67 mM succinate. Table 3, under "succinate 0·67 mM", shows that severe inhibition of oxidation was apparent at the first 10 min reading after adding L-malate, showing that the substrate oxidized during preincubation need not be that of a DPN-dependent enzyme system. The inhibition was of the same order as that shown after pre-incubation with L-malate (Table 3, under "L-malate 5 mM").

TABLE 3. EFFECT OF SUBSTRATES ON INHIBITION OF L-MALATE OXIDATION

Time (min)	Interval oxygen uptake (μl)					
	No substrate control	*d*-tubocurarine (1 mM)	Succinate (0·67 mM) control	*d*-tubocurarine (1 mM)	L-Malate (5 mM) control	*d*-tubocurarine (1 mM)
10	5	7	35	29	33	30
	Mixing equilibration of 5 min after adding L-malate from side-arm compartments					
10	7	10	29	7	27	12
20	11	13	31	12	31	12
30	17	9	23	3	20	1
40	16	7	25	0	19	0

System: As in Table 1. Additions of substrate to the centre compartments are indicated below. L-Malate was added from side-arm compartments 20 min after commencing incubation, including 10 min equilibration, to give a final concentration of 10 mM.

(ii) *Effect on mitochondrial permeability.* Inhibition of pyridine nucleotide-dependent enzyme systems and not of succinoxidase, and protection against inhibition by the addition of DPN, indicated that *d*-tubocurarine and the complex ions led to the loss or inactivation of mitochondrial pyridine nucleotides. Further, the protective effect

of EDTA which is known to stabilize mitochondrial integrity,[16–19] and which appears to act on the mitochondrial membrane,[20] indicated that the inhibitory agents might lead to increased permeability of mitochondrial membranes and thus the loss of pyridine nucleotides.

Mitochondrial permeability may be measured by the rate of reduction of DPN which has been added to mitochondria oxidizing a DPN-dependent substrate.[21–24] The rate of reduction of DPN is regulated by the permeability of mitochondrial membranes to DPN. No effect of 1 mM *d*-tubocurarine or of 0·5 mM *l*-Ru $(phen)_3^{2+}$ could be demonstrated when this system was tested with mitochondria suspended in 0·25 M sucrose or in water. The normal rate of reduction of DPN by water-disrupted mitochondria indicated that under the conditions of these experiments as well as in the manometric assay, neither *d*-tubocurarine nor *l*-Ru $(phen)_3^{2+}$ affected L-malic dehydrogenase activity directly.

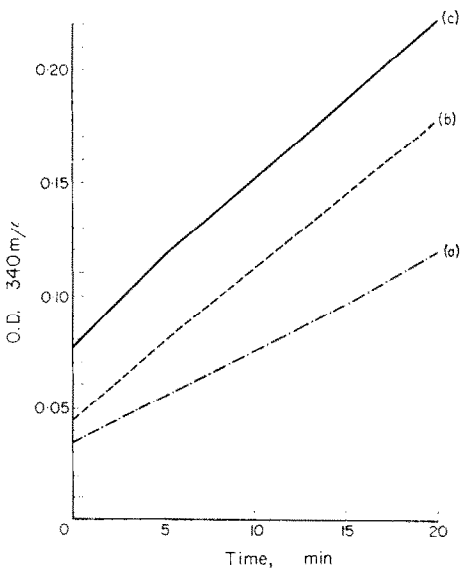

FIG. 1. Reduction of DPN by liver mitochondria in 0·25 M sucrose pre-incubated with: (a) L-malate 10mM; (b) L-malate 10 mM + *d*-tubocurarine 1 mM; (c) no addition.
System: NaK phosphate buffer, pH 7·8, 33 mM; KCl 25 mM; $MgSO_4$ 6·7 mM, L-malate 50 mM; semicarbazide HCl (neutralized) 0·17 M; DPN 1·5 mM; KCN 0·2 mM; enzyme, 50μl 1 per cent mitochondria in 0·25 M sucrose; final volume 3 ml; gas phase air; temperature 22°.

The experimental conditions of such spectrophotometric experiments (cf. Fig. 1) were quite different from those of manometric experiments (cf. Table 1). Furthermore the slow development of inhibition by *d*-tubocurarine or by the complex ions and the dependence upon the oxidation of a substrate indicated that the oxidation–reduction state of the electron transport chain might be a determinant of inhibition. Increased mitochondrial permeability might not be evident except under oxidizing conditions when electrons are moving along the terminal electron transport chain.

Mitochondria re-isolated from manometric experiments in which they were treated with 1 mM *d*-tubocurarine or 0·5 ml *l*-Ru $(phen)_3^{2+}$ were found to be more permeable to DPN than were incubated control mitochondria. This effect was also demonstrated

in the following way. Mitochondria equivalent to 800 mg of fresh rat liver were suspended in 5 ml of 0·25 M sucrose containing, where indicated in Fig. 1, 10 mM L-malate and 1 mM *d*-tubocurarine, and placed in an incubator at 37 ° for 30 min. During the incubation, the temperature of the reaction mixture rose to 32 °. After adding 35 ml of ice-cold 0·25 M sucrose the mitochondria were re-isolated by centrifuging at 34,000 **g** for 10 min at 0 °. The mitochondria were re-suspended in 0·25 M sucrose or in water, and permeability was assessed by the spectrophotometric method.[21–23] Fig. 1 shows that incubation of mitochondria in the presence of *d*-tubocurarine and L-malate increased permeability to DPN. The rate of reduction of DPN, and thus the increase in permeability, due to *d*-tubocurarine with L-malate is about the same as that resulting from incubation of mitochondria without a substrate (Fig. 1). Initial differences in extinction coefficient shown in the curves for L-malate and L-malate plus *d*-tubocurarine in Fig. 1 would be due to reduction of DPN at different rates during the short time between adding the enzyme and taking the first reading as *d*-tubocurarine does not absorb light appreciably at 340 mμ. Mitochondria pre-incubated without substrate invariably had a higher initial extinction coefficient at 340 mμ than those incubated with substrate. Suspension of the pre-incubated mitochondria in water, which abolishes selective semipermeability of the membranes, instead of 0·25 M sucrose, completely removed any difference of the mitochondria treated with *d*-tubocurarine from controls. The effect of *d*-tubocurarine must therefore have been associated with mitochondrial permeability.

Mitochondrial permeability was found to increase under the conditions of manometric experiments. Both *d*-tubocurarine and *l*-Ru $(phen)_3^{2+}$ were observed to stimulate succinate oxidation by mitochondria in 0·25 M sucrose. This indicated the possibility of increased permeability of mitochondrial membranes allowing the loss of mitochondrial DPN, and blocking of the tricarboxylic acid cycle at L-malate thus preventing the production of oxaloacetate, which in low concentration inhibits succinic dehydrogenase specifically.[25–27] Lack of an effect in most experiments and only slight inhibition in others of *d*-tubocurarine on succinate oxidation by mitochondria in water favoured this explanation, as water-disrupted mitochondria would be deficient in DPN and therefore not producing oxaloacetate. Confirmatory evidence was obtained by adding DPN to mitochondria oxidizing succinate in the presence of 1 mM *d*-tubocurarine. DPN added outside intact mitochondria enters slowly unless permeability is increased.[28] Because of the slow penetration of DPN into mitochondria in 0·25 M sucrose, succinate oxidation was only slightly inhibited by adding 0·5 mM DPN alone (Table 4). However, the addition of 1 mM *d*-tubocurarine as well as 0·5 mM DPN led to severe inhibition of the oxidation of succinate by mitochondria in 0·25 M sucrose (Table 4). Table 4 also shows that when mitochondria were disrupted osmotically by suspension in water, DPN alone produced maximum inhibition of succinate oxidation and *d*-tubocurarine did not significantly alter the rate of oxidation when added with or without DPN. Similar results were obtained with 0·5 mM *l*-Ru $(phen)_3^{2+}$ except that the ion itself was inhibitory to succinate oxidation by water-cracked mitochondria. Ru $(phen)_3^{2+}$ stimulated succinate oxidation by mitochondria in 0·25 M sucrose when added alone and facilitated inhibition by DPN (Table 4).

The addition of 0·4 mM $CaCl_2$ to a system oxidizing succinate by mitochondria produced a pattern of events similar to those resulting from *d*-tubocurarine.[28] Ca^{2+}

stimulated succinate oxidation by mitochondria in 0·25 M sucrose and facilitated the penetration by added DPN and consequent inhibition of oxidation (Table 4). Suspension of mitochondria in water also removed the effect of Ca^{2+}. The inhibition of succinate oxidation by mitochondria in 0·25 M sucrose resulting from Ca^{2+} plus DPN was not significantly increased by adding either 1 mM *d*-tubocurarine or 0·5 mM *l*-Ru $(phen)_3^{2+}$ as shown in Table 4.

TABLE 4. EFFECT OF *d*-TUBOCURARINE, CALCIUM AND DPN ON SUCCINATE OXIDATION

Additions	Oxygen uptake (μl/30 min)			
	Sucrose medium		Water medium	
	No DPN	DPN 0·5mM	No DPN	DPN 0·5 mM
None	161	137	132	42
d-Tubocurarine (1 mM)	220	53	112	34
l-Ru $(phen)_3^{2+}$ (0·5 mM)	228	30	22	27
$CaCl_2$ (0·4 mM)	212	37	134	30
d-Tubocurarine (1 mM) + $CaCl_2$ (0·4 mM)	216	35	—	—
l-Ru $(phen)_3^{2+}$ (0·5 mM) + $CaCl_2$ (0·4 mM)	217	31	—	—

System: As in Table 1 + succinate 10 mM. Mitochondria equivalent to 50 mg of fresh rat liver were added in 0·5 ml of 0·25 M sucrose or water.

The stimulatory effect of *d*-tubocurarine on succinate oxidation and inhibition in the presence of DPN was present when mitochondria were isolated from homogenates prepared in 0·88 M sucrose instead of the usual 0·25 M sucrose and resuspended in 0·25 M sucrose. However, addition of sucrose to the Warburg flask to give a final concentration of 0·5 M eliminated both the capacity of the succinoxidase system to be stimulated by *d*-tubocurarine or inhibited by *d*-tubocurarine plus DPN.

Clearly *d*-tubocurarine and *l*-Ru $(phen)_3^{2+}$, like Ca^{2+}, increased the permeability of mitochondrial membranes to DPN.

(d) *Uptake of* d-*tubocurarine by mitochondria*

(i) *Absorption of light at* 260 mμ *by, and* d-*tubocurarine content of, mitochondrial extracts.* In order to determine whether oxidized pyridine nucleotides were lost from mitochondria during incubation with *d*-tubocurarine, mitochondria were recovered from Warburg flasks after respiratory experiments and extracted with 5 per cent TCA as follows:

The contents of Warburg flasks were transferred quantitatively to nylon centrifuge tubes using a Pasteur pipette and washing the flasks out thoroughly with ice-cold, 0·25 M sucrose. The volume in the centrifuge tube was made to 40 ml with ice-cold 0·25 M sucrose, and mitochondria were re-isolated by centrifuging at 34,000 g for 10 min at 0 °. The supernatant was discarded and the inside of the tubes dried with filter paper taking care not to touch the mitochondrial pellet. Mitochondria were triturated with a glass rod in 5 ml of 5 per cent TCA, and allowed to stand at room temperature for 10 min. The contents of the nylon tubes were transferred to glass centrifuge tubes and centrifuged at 2000 rev/min for 10 min in an M.S.E. Multiplex centrifuge. The supernatant was collected, neutralized with 2 N NaOH, and made to

6 ml. Absorption of light by the extracts was read at 260 mμ with a light path of 1 cm against a reagent blank of TCA, NaOH and water.

It was clear from the experiments recorded in Table 5, that incubation of mitochondria in the presence of an oxidizable substrate and *d*-tubocurarine increased the TCA extractable-material which absorbed light at 260 mμ. *d*-Tubocurarine was found to absorb light at 260 mμ and concomitant measurement of the *d*-tubocurarine content of the TCA extracts of mitochondria showed that much at least of the increase in absorption at 260 mμ by such extracts was due to uptake of *d*-tubocurarine by the mitochondria (Table 5). Aliquots of 3 ml were taken from the 5 per cent TCA extracts and developed for the measurement of *d*-tubocurarine at 430 mμ by the method of Pride and Smith.[12] It was realized that this method for *d*-tubocurarine analysis was not highly accurate under the conditions of these experiments, but the method was sufficiently accurate and dependable for the required purpose.

TABLE 5. UPTAKE OF *d*-TUBOCURARINE BY MITOCHONDRIA

Additions	Extinction coefficient				*d*-Tubocurarine uptake (μM/flask)
	Control		*d*-Tubocurarine (1 mM)		
	260 mμ	430 mμ	260 mμ	430 mμ	
None	0·126	0·012	0·129	0·012	0·00
L-malate (10 mM)	0·202	0·011	0·414	0·037	0·34
Succinate (10 mM)	0·148	0·008	0·197	0·023	0·14
Succinate (10 mM) + amytal (1·8 mM)	0·190	0·010	0·116	0·013	0·00
Succinate (10 mM) + DPN (0·5 mM)	—	—	0·298	0·026	0·18
Succinate (10 mM) + DPN (0·5 mM) + amytal (1·8 mM)	—	—	0·137	0·014	0·00

System: As in Table 1. Mitochondria equivalent to 100 mg of fresh liver were added in 0·5 ml of 0·25 M sucrose. Incubation time was 20 min after 10 min equlibration. Mitochondria were re-isolated, extracted with 5 per cent TCA and analysed for *d*-tubocurarine as described in the text.

d-Tubocurarine uptake was calculated from E_{430} readings with correction for E_{430} of mitochondrial extracts in absence of *d*-tubocurarine.

Table 5 shows that mitochondria did not take up *d*-tubocurarine when incubated without added substrate. However, incubation with either L-malate or succinate led to an appreciable uptake of *d*-tubocurarine as measured by extinction coefficients at both 260 mμ and 430 mμ. The uptake of *d*-tubocurarine was consistently greater from incubation with L-malate than with succinate (Table 5). Incubation was found to be necessary for *d*-tubocurarine uptake to occur, as immediate extracts of mitochondria which had been added to reaction mixtures as in Table 5 containing L-malate or succinate and *d*-tubocurarine were not different in absorption either at 260 mμ or at 430 mμ from other mitochondria in the absence of *d*-tubocurarine.

The presence of 1 mM EDTA in the respiratory system did not affect the uptake of *d*-tubocurarine by mitochondria.

Oxidation of L-malate was accompanied by a peak of uptake of *d*-tubocurarine by mitochondria at about 30 min after commencing incubation, including 10 min equilibration. Longer incubation led to the loss of material absorbing light at 260 mμ but

less loss of *d*-tubocurarine as measured at 430 mμ. For example, the experiments comprising group 1 of Table 6 showed that after 50 min incubation with L-malate and *d*-tubocurarine, mitochondrial extracts had an extinction coefficient at 260 mμ of 0·218, as compared with 0·414 after 30 min incubation as shown in Table 5.

d-Tubocurarine uptake by mitochondria during the oxidation of succinate might be related to the reduction and subsequent re-oxidation of pyridine nucleotides, even though the succinoxidase system is not dependent upon pyridine nucleotides for electron transport. Chance and Hollunger[29] have shown that mitochondrial pyridine nucleotides are reduced rapidly during the oxidation of succinate. The uptake of *d*-tubocurarine by mitochondria accompanying succinate oxidation was found to be prevented by adding 1·8 mM Na amytal, which inhibits the re-oxidation of reduced pyridine nucleotides[30] (Table 5). Further it was shown that the increased absorption at 260 mμ resulting from incubation of mitochondria with succinate, DPN and *d*-tubocurarine, and the *d*-tubocurarine uptake as measured at 430 mμ are prevented by the addition of amytal (Table 5).

(ii) *Localization of* d-*tubocurarine in mitochondria.* The coincidence of *d*-tubocurarine uptake with activity of the succinoxidase system, which is associated with mitochondrial membranes,[31] and the effect of *d*-tubocurarine on mitochondrial membrane permeability, indicated that the uptake of *d*-tubocurarine might be by some component of the mitochondrial membrane.

Succinate was oxidized by mitochondria which had been suspended in water and stood for 1 hr at 0 ° to ensure water-cracking. The mitochondrial fragments were re-isolated by transferring the Warburg flask contents quantitatively to centrifuge tubes, and centrifuging at 45,000 **g** for 20 min at 0 °. The mitochondrial pellet was then analysed for *d*-tubocurarine and for material absorbing at 260 mμ.

TABLE 6. LOCALIZATION OF *d*-TUBOCURARINE IN MITOCHONDRIA

Group	Additions	Extinction coefficient			
		Control		*d*-Tubocurarine (1 mM)	
		260 mμ	430 mμ	260 mμ	430 mμ
1	L-Malate (10 mM)	0·174	0·010	0·218	0·027
2	L-Malate (10 mM)	0·044	0·011	0·132	0·024
3	Succinate (10 mM)	0·083	0·010	0·116	0·016

System: As in Table 1. Mitochondria equivalent to 100 mg of fresh liver were added in 0·5 ml of 0·25 M sucrose for L-malate oxidation and 0·5 ml of water for succinate oxidation.

Incubation times were 40 min for L-malate oxidation and 30 min for succinate oxidation after 10 min equilibration, respectively. Mitochondria were re-isolated as described in text.

Table 6 shows that very little uptake of *d*-tubocurarine occurred during succinate oxidation by water-cracked mitochondria. However, a slight uptake was recorded in all experiments.

This aspect was further investigated by oxidizing L-malate with mitochondria in 0·25 M sucrose in the presence of *d*-tubocurarine. The contents of the Warburg flasks were washed into centrifuge tubes with ice-cold 0·25 M sucrose or water. Water or

0·25 M sucrose was added to 40 ml and the centrifuge tubes stood for 90 min at 0–1 ° to allow osmotic disruption of those mitochondria which had been suspended in water. The tubes were centrifuged at 45,000 **g** for 20 min at 0 ° and the mitochondrial pellet analysed for material absorbing at 260 mμ and for *d*-tubocurarine as described.

Suspension of the mitochondria in water resulted in a great loss of 260 mμ absorbing material (group 2, Table 6), as compared with mitochondria suspended in 0·25 M sucrose (group 1, Table 6), but almost no loss of *d*-tubocurarine. This suggested that *d*-tubocurarine was bound to the membranous portion of mitochondria.

DISCUSSION

The neuromuscular blocking agents, *d*-tubocurarine, and the complex ions, *l*- or *d*-Ru (phen)$_3^{2+}$ and Ru (trpy)$_2^{2+}$, have been shown to affect the metabolism of liver mitochondria in a manner similar to the effect of pyrrolizidine alkaloids,[2] which are also neuromuscular blocking agents.[1] Each of these compounds inhibits the oxidations of substrates by mitochondrial enzyme systems which require pyridine nucleotides for electron transfer, and stimulates the oxidation of succinate by mitochondria in 0·25 M sucrose.

Inhibition of oxidations by *d*-tubocurarine or *l*-Ru (phen)$_3^{2+}$, the complex ion which was chosen for detailed study, is prevented by the addition of DPN to the reaction mixture of Warburg flasks. Some protection against inhibition is afforded also by EDTA. In both respects, the effects of *d*-tubocurarine and *l*-Ru (phen)$_3^{2+}$ resemble that of the pyrrolizidine alkaloids.

Neither *d*-tubocurarine nor *l*-Ru (phen)$_3^{2+}$ had a direct effect on the activity of L-malic dehydrogenase under the conditions of either manometric or spectrophotometric experiments. *d*-Tubocurarine also was without effect on the succinoxidase system when the permeability of mitochondria was increased by suspension in water instead of in 0·25 M sucrose. In these respects, too, the compounds resemble pyrrolizidine alkaloids. However, unlike *d*-tubocurarine or the pyrrolizidine alkaloids, *l*-Ru (phen)$_3^{2+}$ inhibits succinoxidase activity of mitochondria suspended in water.

The effects of *d*-tubocurarine and of *l*-Ru (phen)$_3^{2+}$, like the effects of pyrrolizidine alkaloids, on oxidative metabolism of liver mitochondria suspended in 0·25 M sucrose are probably due to the agents increasing the permeability of mitochondrial membranes, and thus accelerating the loss of mitochondrial pyridine nucleotides. Loss of pyridine nucleotides from mitochondria inactivates those enzyme systems which are dependent upon pyridine nucleotides for electron transfer and stimulates succinoxidase activity by blocking the formation of oxaloacetate, which inhibits succinic dehydrogenase, at the DPN-requiring step catalysed by L-malic dehydrogenase.[27]

For *d*-tubocurarine and *l*-Ru (phen)$_3^{2+}$ to increase mitochondrial permeability and inhibit pyridine nucleotide-dependent enzyme systems, the mitochondria need to be oxidizing an added substrate. The substrate need not be one oxidized by enzymes which require pyridine nucleotides. The dependence upon the presence of a substrate for the development of inhibition, indicated that the effects of *d*-tubocurarine and *l*-Ru (phen)$_3^{2+}$ might be linked to the state of oxidation and reduction of components of the terminal electron transport chain. As neither agent inhibits cytochrome oxidase or the succinoxidase system, the most likely point at which they might act appeared to be the pyridine nucleotide level. The ability of succinate oxidation to predispose mitochondria to inhibition of L-malate oxidation is not at variance with this

possibility, as it is known[29] that mitochondrial pyridine nucleotides are rapidly reduced when succinate is oxidized, although the succinoxidase system does not require the coenzymes for electron transport.

Mitochondria incubated with *d*-tubocurarine have been found to take up appreciable quantities of the alkaloid. A portion, at least, of the *d*-tubocurarine appears to become localized in the insoluble, membranous fractions of mitochondria. The uptake requires the oxidation of an added substrate and is greater for the DPN-dependent oxidation of L-malate than for succinate oxidation. However, *d*-tubocurarine uptake does occur when succinate is oxidized, and the addition of amytal prevents the uptake, although succinate oxidation is not altered. Amytal inhibits the re-oxidation of reduced DPN[30]. The uptake of *d*-tubocurarine by mitochondria appears then to be associated with the re-oxidation of reduced pyridine nucleotide.

It is possible that the reactions reponsible for the uptake of *d*-tubocurarine are also concerned in increasing mitochondrial permeability and causing the loss of pyridine nucleotides from dependent enzyme systems. Further evidence on this point is supplied by the experiments in which *d*-tubocurarine was incubated with mitochondria which were oxidizing succinate in the presence of added DPN. TCA extracts of such mitochondria absorbed more light at 260 mμ than could be accounted for by the uptake of *d*-tubocurarine and so was probably due to the uptake of added DPN by the mitochondria. Both the uptake of added DPN and of *d*-tubocurarine were prevented by adding amytal to this system, showing that the re-oxidation of reduced DPN was in some manner involved in producing both increased mitochondrial permeability to DPN and to the uptake of *d*-tubocurarine.

Following an initial increase due to uptake of *d*-tubocurarine, the concentration of material absorbing light at 260 mμ in TCA extracts of mitochondria falls progressively with the period of incubation. The loss of 260 mμ absorbing material is not accounted for by the loss of *d*-tubocurarine and would seem likely to be due to the loss of oxidized pyridine nucleotides as was found to occur with the pyrrolizidine alkaloids.[2] A study of the effects of *d*-tubocurarine, *l*-Ru (phen)$_3^{2+}$ and the pyrrolizidine alkaloids on mitochondrial nucleotides will be presented in a subsequent paper.

In the case of pyrrolizidine alkaloids, it was found that the nitrogen atom of the cyclic base was involved in producing inhibitory effects both on liver mitochondrial metabolism[2] and on the transmission of impulses across neuromuscular junctions.[1] *d*-Tubocurarine is a neuromuscular blocking agent by nature of its two quaternary nitrogen atoms and has now been shown to affect liver mitochondrial oxidations in the same manner as do the pyrrolizidine alkaloids.[1] The complex ions are neuromuscular blocking agents due to the charge which they carry[3-7] and have also been found to influence mitochondrial metabolism in a manner similar to the pyrrolizidine alkaloids and to *d*-tubocurarine. Under appropriate oxidizing conditions, the three groups of agents increase mitochondrial permeability to the movement of pyridine nucleotides. The question of whether this is due to a general increase in membrane permeability or a specific increase in permeability to pyridine nucleotides is being investigated. The effect of the agents at neuromuscular junctions is also thought to be at the membrane level. Lower effective concentrations at the neuromuscular junction than with liver mitochondria may be due to greater accessibility or susceptibility to the drugs or to unsuitability of the *in vitro* conditions for optimal demonstration of the effects on liver mitochondria.

d-Tubocurarine, pyrrolizidine alkaloids and the complex ions possibly produce their effects by the same mechanism on liver mitochondria and on neuromuscular junctions. This mechanism may be related to the charge carried by the nitrogen atoms of the alkaloids in the same way as the charge on the complex ions is effective and may be associated with derangement of membrane functions by such charge.

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