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MODIFICATION OF NICOTINE TOXICITY BY PRETREATMENT WITH DIFFERENT DRUGS

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Abstract—Sub-lethal doses of nicotine have been shown to protect mice from the lethal effects of subsequently administered doses of the same drug. The evidence suggests that a metabolic product of nicotine is responsible for the protective action. In an attempt to identify this metabolite it was shown that cotinine had no protective action but that nicotine *N*-oxide and also the reduction product of cotinine both conferred some protection. The pharmacological basis of the protective effect is not clear but it may involve ganglion-blocking activity; the ganglion-blocking drugs hexamethonium and pempidine also protected mice from the lethal effects of nicotine.

IT HAS been shown by Hazard and Savini[1] that sub-lethal doses of certain nicotinic drugs when administered to rats gave some degree of protection against the lethal effects of subsequent higher doses of the same, or different, nicotinic compounds. These authors distinguished between "autoprotection" when the same drug was used in both cases, and "heteroprotection" when the drug used for premedication differed from that subsequently used as the toxic challenge. Nicotine was the most effective drug studied and it was shown that the time interval which elapsed between the administration of the two drugs was of considerable importance. For example, an intravenous (i.v.) dose of 2 mg/kg of nicotine (the maximum dose which could be used without the risk of death) gave almost complete protection against the lethal effects of 10 mg/kg of nicotine (twice the LD_{50}) provided the second dose of nicotine was given within the period 30–110 min after the administration of the first dose. Lobeline and procaine methiodide, whilst not exhibiting autoprotection, gave heteroprotection against nicotine.

In the course of other work Barrass *et al.*[2] showed that pretreatment of mice with a dose of nicotine corresponding to approximately half an LD_{50} raised, by about eight times, the LD_{50} of nicotine administered 40 min later. Since the time interval between the two doses was of considerable significance it was thought likely that a metabolite of nicotine was involved, and indeed administration of the metabolic inhibitor proadifen (SKF 525A) prior to the first dose of nicotine reduced the protective effect significantly. Ethyl β-dimethylaminopropionate methiodide, another nicotinic agent, showed no autoprotection, neither was there any heteroprotection between nicotine and this drug.

In the investigation reported here a wide range of drugs was studied and an attempt was made to identify the nicotine metabolite which might be responsible for the protective effect.

METHODS

Drugs

Cotinine was synthesised from nicotine by the method of McKennis *et al.*[3] Procaine methiodide was prepared by the method of Jensen *et al.*[4] Ethyl β-dimethyl amino-propionate methiodide was prepared by the method of Barrass *et al.*[2] 2-(Bromo-phenoxyethyl)trimethylammonium bromide was synthesised by the method of Hey.[5] Nicotine-*N*-oxide was a gift from Professor E. Boyland (Chester Beatty Research Institute) and is gratefully acknowledged. With the exception of cotinine methiodide and the reduction product of cotinine, the preparation of which is given below, the remainder of the compounds were purchased from commercial sources.

Cotinine methiodide. Cotinine (3 g) and methyl iodide (3g) were refluxed 1 hr in methanol (10 ml). Ethyl acetate was added to the cooled mixture and the precipitated oil caused to crystallise by cooling. Recrystallisation from ethyl alcohol gave the pure compound in 86 per cent yield, m.p. 154–156°. (Found: C, 41·6; H, 5·1; N, 8·55%. $C_{11}H_{15}IN_2O$ requires C, 41·5; H, 4·75; N, 8·8%.)

Reduction of cotinine. A slurry of lithium aluminium hydride (1·3 g) in dry ether (20 ml) was added dropwise over 30 min to a stirred mixture of cotinine (10·9 g) in dry ether. The mixture was stirred at room temperature for 5 hr, decomposed by the careful addition of wet ether, filtered and dried over anhydrous magnesium sulphate. The drying agent was removed by filtration and the solvent removed in vacuo without application of external heat to give a pale yellow oil (5 g) which was used as such for the toxicological studies. Examination of this oil by thin layer chromatography revealed that it was essentially one compound, although faint traces of nicotine and cotinine were present. All attempts to purify this material or to prepare solid derivatives were unsuccessful. The biological activity of this preparation was lost over a period of 7 days at 0°.

Toxicological studies

Female albino mice of the Porton strain in a weight range of 18–25 g were used. All drugs were injected either i.v. into a tail vein or by the intraperitoneal (i.p.) route. In the preliminary experiments designed to obtain approximate estimates of the LD_{50}, groups of only two animals were used but all the LD_{50} values quoted in this paper were obtained from experiments in which at least four groups each containing five mice were used. The ratio between doses was 1:1·5 and LD_{50} values were calculated using the method of probit analysis.[6]

RESULTS

The experiments described in this paper were carried out over a period of approximately 2 yr. The intravenous LD_{50} of nicotine hydrogen tartrate to mice was determined at intervals and whilst the figures varied somewhat over this period these variations were not sufficiently large to warrant using different LD_{50}'s in the calculation of indices of protection. An overall LD_{50} for nicotine was therefore calculated, using results from all the individual determinations (27 in all); this figure was 2·03 mg/kg with 95 per cent confidence limits of 1·64–2·49 mg/kg.

Table 1 lists the results of experiments in which the LD_{50}'s of nicotine were determined after pretreatment with 0·8 mg/kg of the same drug given previously at various

TABLE 1. THE INTRAVENOUS TOXICITY OF NICOTINE TO MALE ALBINO MICE PRETREATED WITH 0·8 mg/kg OF THE SAME DRUG (i.v.)

Interval between pretreatment and LD_{50} determination (min)	LD_{50} (mg/kg with 95 per cent limits)	Protection index*
5	20·8 (17·0–25·3)	10·4
17	17·6 (12·5–25·7)	8·8
28	10·1 (6·9–15·8)	5·0
50	13·8 (9·8–35·5)	6·9

* $\frac{LD_{50} \text{ after pretreatment}}{\text{Control } LD_{50} \text{ (i.e. 2·0 mg/kg)}}$

times. This pretreatment resulted in significantly higher LD_{50}'s at all times tested between 5 and 40 min after the first sub-lethal dose of nicotine.

Table 2 shows the effect of the metabolic inhibitor proadifen (SKF 525A) on this pretreatment. It was found to reduce the protective effect of the first, sub-lethal, dose of nicotine to statistically nonsignificant levels.

A number of other drugs covering a range of pharmacological activities were

TABLE 2. THE INTRAVENOUS TOXICITY OF NICOTINE TO MALE ALBINO MICE PRETREATED WITH 0·8 mg/kg OF THE SAME DRUG (i.v.). SKF 525A (50 mg/kg i.p.) HAVING BEEN GIVEN 40 min BEFORE THE PRETREATMENT

Interval between pretreatment and LD_{50} determination (min)	LD_{50} (mg/kg with 95 per cent limits)	Protection index
No pretreatment	3·1 (2·4–4·0)	1·5
5	4·0 (2·8–6·1)	2·0
40	4·2 (3·1–5·8)	2·1

tested for their ability to influence the toxicity of nicotine. The results are listed in Table 3. Pretreatment with the ganglion-blocking drugs hexamethonium and pempidine gave a significant degree of protection as also did pretreatment with the α-adrenergic blocking agent phentolamine. The only other drug which gave a significant degree of protection was the nicotinic drug lobeline.

In another series of experiments the effect of pretreatment with nicotine on the toxicity of some other drugs was studied. The results are given in Table 4. Nicotine did not afford any protection against the lethal effects of any of these drugs and in fact the LD_{50} of carbachol was decreased by pretreatment with nicotine.

The final series of experiments was concerned with investigating the effects of possible nicotine metabolites, and derivatives, on the toxicity of nicotine. The compounds investigated were cotinine and its methiodide, nicotine *N*-oxide, and the reduction product of cotinine; the results are listed in Table 5. Neither cotinine nor its methiodide affected the LD_{50} of nicotine, but the reduction product of cotinine was

TABLE 3. THE INTRAVENOUS TOXICITY OF NICOTINE TO MALE ALBINO MICE PRETREATED WITH OTHER DRUGS

Drug	Main type of pharmacological action	Dose (mg/kg)	Route of administration	Interval between pretreatment and LD_{50} determination (min)	LD_{50} nicotine (mg/kg with 95 per cent limits)
Hexamethonium bromide	Ganglion blockade	30	i.p.	20	10·7* (6·5–17·5)
Decamethonium bromide	Depolarising neuromuscular blockade	2	i.p.	20	2·0 (1·0–3·4)
Pempidine tartrate	Ganglion blockade	23	i.p.	20	32·2* (29·5–36·8)
Tubocurarine chloride	Non-depolarising neuromuscular blockade	0·3	i.p.	20	2·5 (1·6–4·4)
Phentolamine methane sulphonate	α-adrenoreceptor blockade	25	i.p.	30	4·6* (3·6–54)
Atropine sulphate	Anticholinergic	17	i.p.	30	2·8 (2·1–3·7)
Carbamoylcholine chloride	Nicotinic and Muscarinic	0·17	i.v.	5 40	1·7 (1·2–2·5) 2·0 (1·3–3·2)
Procaine methiodide	Local anaesthetic	11	i.v.	40	2·3 (1·7–3·2)
Lobeline hydrochloride	Nicotinic	4	i.v.	5 40	11·7* (8·5–16·2) 4·0 (2·1–6·2)
Ethyl β dimethylpropionate methiodide	Nicotinic	0·2	i.v.	40	1·7 (1·4–2·1)

* LD_{50} significantly different from that of nicotine alone.

TABLE 4. THE EFFECT OF PRETREATMENT WITH NICOTINE HYDROGEN TARTRATE (0·8 mg/kg) ON THE TOXICITY TO MALE ALBINO MICE OF SOME OTHER DRUGS

Drug	Main type of pharmacological action	LD_{50} (mg/kg with 95 per cent limits)	Interval between pretreatment and LD_{50} determination (min)	LD_{50} after nicotine pretreatment (mg/kg with 95 per cent limits)
Carbamoylcholine chloride	Nicotinic and muscarinic	0·42 (0·39–0·46)	5	*0·26 (0·18–0·37)
			40	0·35 (0·26–0·48)
Decamethonium bromide	Depolarising neuromuscular blockade	0·9 (0·56–1·2)	40	1·0 (0·85–1·3)
Dimethylphenyl-piperazinium iodide	Nicotinic	1·2 (0·8–1·7)	40	1·4 (0·93–1·6)
Succinylcholine chloride	Depolarising neuromuscular blockade	0·59 (0·41–0·86)	40	0·54 (0·46–0·74)
Tetramethylammonium iodide	Nicotinic	3·5 (2·6–4·8)	40	3·3 (2·2–4·5)
Ethyl β-dimethylaminopropionate methiodide	Nicotinic	0·43 (0·35–0·53)	40	0·40 (0·33–0·48)
2-Dimethylaminoethyl-*m*-bromophenyl ether methobromide	Nicotinic	1·1 (0·74–1·8)	40	0·76 (0·54–1·1)
Lobeline hydrochloride	Nicotinic	7·8 (6·7–9·2)	5	10·8 (7·8–15·0)
			40	9·9 (8·5–11·8)

* LD_{50} significantly changed by nicotine pretreatment.

highly effective, elevating the LD_{50} of nicotine some nine times when given 5 min before nicotine; after 40 min the protection was still six-fold. Nicotine *N*-oxide was also effective, elevating the LD_{50} of nicotine nine times when given 40 min before the nicotine; when the time interval between the doses of drugs was reduced to 5 min, the protection effect was 4 to 5, this latter figure being only approximate due to lack of material.

The effect of pretreatment with nicotine on the toxicity of the reduction product of cotinine was also tested. The results, given in Table 6, show that nicotine given

TABLE 5. THE INTRAVENOUS TOXICITY OF NICOTINE TO MALE ALBINO MICE PRETREATED INTRAVENOUSLY WITH POSSIBLE NICOTINE METABOLITES

Drug	Dose (mg/kg)	Interval between pretreatment and LD_{50} determination (min)	LD_{50} (mg/kg with 95 per cent limits)	Protection index
Cotinine	0·8	5	1·8 (1·2–2·7)	0·9
		40	1·7 (1·1–2·4)	0·85
Cotinine methiodide	0·8	5	2·2 (1·0–3·7)	1·1
		40	1·9 (1·3–2·6)	0·95
Reduction product of cotinine	0·8	5	18·8 (16·0–22·1)	9·4
		40	12·6 (8·6–18·0)	6·3
Nicotine *N*-oxide, 2HCl	28·5	5	*4·5	2·2
		40	17·7 (15·1–20·9)	8·85

* Approximate value only. Insufficient material for accurate LD_{50} determination.

TABLE 6. THE EFFECT OF PRETREATMENT WITH NICOTINE HYDROGEN TARTRATE (0·8mg/kg) ON THE INTRAVENOUS TOXICITY TO MALE ALBINO MICE OF THE REDUCTION PRODUCT OF COTININE

LD_{50} of reduction product of cotinine (mg/kg with 95 per cent limits)	Interval between pre-treatment and LD_0 determination (min)	LD_{50} after nicotine pretreatment (mg/kg with 95 per cent limits)
20·1 (15·6–25·5)	5	33·6 (24·5–45·7)
	40	36·3 (26·4–49·8)

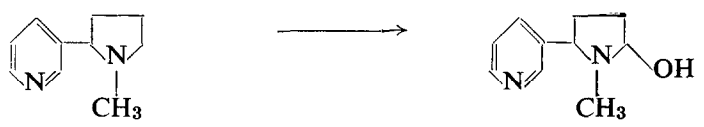
5 min before the reduction product had no significant effect on its LD_{50} but when it was given 40 min before the reduction product there was a significant elevation of the LD_{50} with a protective index of 1·8.

DISCUSSION

The results reported confirm earlier observations[1, 2] that sub-lethal doses of nicotine will protect mice against the lethal effects of subsequently administered doses of nicotine. They also strongly support the suggestion, made by Barrass *et al.*[2] that a metabolite of nicotine is involved in this process. McKennis *et al.*[3] have shown that in the dog, nicotine (I) is metabolised to cotinine (II) and they claimed that cotinine is the first isolable metabolite of nicotine.

I II

However, mice pretreated with cotinine were not protected against subsequent lethal doses of nicotine given either immediately after the pretreatment or 40 min later. This experiment shows quite clearly that cotinine is not the active metabolite nor is it on a metabolic pathway leading to the active metabolite. This active metabolite therefore must either be on a parallel metabolic pathway or be a precursor of cotinine.



III

Hucker *et al.*[7] have proposed that the metabolism of nicotine proceeds initially by a ring hydroxylation to form hydroxynicotine (III) and this is then converted into cotinine. These workers showed that metabolism of nicotine in liver homogenates gave a compound which was not cotinine and which gave characteristic aldehyde reactions; they suggested this was III. It seemed reasonable therefore to try to prepare III and to study its biological properties. Lithium aluminium hydride reduction of cotinine[7] gave a material which was highly effective in protecting mice against the lethal effects of nicotine, moreover it exerted its protective effect immediately. In agreement with previous work[7] we found that this compound was extremely unstable, as could be predicted from its structure as a carbinolamine. All attempts to purify it or to make solid derivatives led to the formation of insoluble polymeric compounds. Investigation of the freshly prepared material by thin layer chromatography indicated that it was essentially one compound although two faint spots corresponding to nicotine and cotinine respectively were apparent when the plates were examined carefully.

Nicotine *N*-oxide also conferred some degree of protection against subsequent lethal doses of nicotine, but in contrast to nicotine and hydroxynicotine, the effect of the *N*-oxide was slow to appear, being maximal about 40 min after administration. It is possible that nicotine *N*-oxide is converted into hydroxynicotine in the body but there was insufficient material to carry out the additional experiments required to establish this point.

These experiments do not provide any explanation of the pharmacological basis of this protective effect. Hazard and Savini[1] considered that it was due to the inhibitory action, secondary to the excitatory phase, of nicotine at the autonomic ganglion. The fact that ganglion blocking drugs such as hexamethonium and nicotinic drugs such as lobeline also protect against nicotine poisoning appears to support this contention. It is difficult, however, to see why this effect seems to be so specific and in particular why the toxicities of nicotinic drugs other than nicotine itself are not similarly modified by pre-treatment with the nicotine metabolite.

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