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IN VITRO HEPATIC AND EXTRA-HEPATIC REDUCTION OF (−)-NICOTINE-1′-*N*-OXIDE IN RATS

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Abstract—The anaerobic reduction of (−)-nicotine-1′-*N*-oxide was studied in various tissues of the rat. Gas–liquid chromatography was employed in the quantitative determination of unchanged drug and its metabolites. With the exception of blood and brain most tissues reduced (−)-nicotine-1′-*N*-oxide in the following order: liver > small intestine > kidney > heart > lung. The predominant cellular sites for reduction of the *N*-oxide were the microsomes and soluble fraction. The reductases were non-specific with regard to their requirement for NADH and NADPH although the latter was more effective. Air or boiling abolished all the reducing activity of the reductases in all fractions studied. SKF525-A and EDTA caused partial inhibition whereas CO or KCN effected almost complete inhibition. Flavins and Phenobarbital enhanced the activity of the (−)-nicotine-1-*N*-oxide reductase(s). It was concluded that (−)-nicotine-1′-oxide reductase(s) could be flavoproteins linked to P-450 and/or to NADPH and appear to be different from other known reductases.

Drug *N*-oxides are readily reduced chemically to the corresponding amines. Recently enzymatic reduction of some of these compounds has been reported in several species and tissues [1]. Studies in man [2] have shown that extensive reduction of (−)-nicotine-1′-*N*-oxide occurred after its oral administration, whereas negligible reduction was effected following the intravenous route. Based on these findings it was hypothesized that intestinal factors, particularly the microflora of the gut, could have been the underlying cause of this reduction. More recently, Booth and Boyland [3] demonstrated significant *in vitro* anaerobic reduction of the two diastereoisomers of (−)-nicotine-1′-*N*-oxide by guinea pig liver preparations, thus implicating another site for the reduction of these *N*-oxides.

The present study was undertaken to gain a better understanding of the role of the liver and other tissues in the reduction of the drug. A preliminary account of some of this work has been reported [4].

METHODS

Materials. Glucose-6-phosphate (G-6-P), nicotinamide, reduced nicotinamide-adenine dinucleotide (NADH), nicotinamide-adenine dinucleotide phosphate (NADP), G-6-P-dehydrogenase, flavin-mononucleotide (FMN) and flavin-adenine dinucleotide (FAD) were purchased from Boehringer & Soehne, GmbH, Mannhein, W. Germany. Riboflavin was obtained from Sigma Chemicals Co., St. Louis, Missouri, U.S.A. *B*-Diethylaminoethyldiphenylpropylacetate HCl (SKF 525-A) is a product of Smith, Kline & French Laboratories, Philadelphia, U.S.A. (−)-Nicotine hydrogen (−)-tartrate was obtained from BDH Ltd, England. (−)-Nicotine-1′-*N*-oxide was synthesized according to Taylor and Boyer [5]. Titanous chloride solution, 30 per cent w/v, was purchased from May & Baker, Ltd, Dagenham, England. Other chemicals were analytical grades.

Animals and tissue preparations. Male Wistar albino rats weighing 250–450 g were maintained on standard pellets (Dixons diet 86) and had free access to water. All tissue preparations were made at 0–5°. The animals were killed by decapitation, and tissues were excised immediately and washed with ice-cold isotonic Tris–KCl buffer (pH 7·4), blotted, weighed and then homogenized with 3 parts (w/v) of buffer in a Potter–Elvehjem homogenizer having a Teflon® pestle. Intestinal tissue was homogenized in an Ultra Turrax homogenizer.

Tissue supernatant free from cell debris and nuclei was prepared by centrifuging the homogenate at 1000 *g* for 20 min in a refrigerated Sorvall Angle Centrifuge RC2B. For preparations where mitochondria were removed the homogenate was centrifuged at 10,000 *g* for 20 min.

Mitochondria were likewise obtained by centrifuging the 1000 *g* supernatant at 10,000 *g* for 20 min. The 10,000 *g* supernatant was further centrifuged at 144,000 *g* for 1 hr in a Measuring and Scientific Equipment Ltd Superspeed 50 Preparative Ultracentrifuge to sediment microsomes. The clear supernatant phase thus prepared constituted the soluble fraction. The mitochondrial and microsomal pellets were each suspended in original volumes of buffer and the centrifugation repeated at the appropriate speeds. The washed

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sediments were then resuspended in buffer or, whenever indicated, in soluble fraction so that 3 ml of suspension were equivalent to 1 g tissue.

Incubation systems. The incubation mixtures and conditions of incubation are described in the respective tables and figures. The anaerobic experiments under N_2 were carried out in Thunberg tubes. For experiments using CO as the gas phase Erlenmeyer flasks fitted with a three-way glass stopcock stoppers were employed. The tubes containing the incubation mixtures were four times alternatively evacuated *in vacuo* and flushed with either gas, as the case may be, which was deoxygenated completely by passing it through a solution containing 0·1% 2-anthraquinone sodium sulfonate, 1% sodium dithionite and 1% sodium hydroxide. The incubation mixtures were preincubated for 10 min at 37° in a water bath with constant shaking (100 strokes/min) before starting the reactions by the addition of the substrate, and then incubated further for the various specified time intervals indicated in the tables. The reactions were terminated with 0·5 ml 2 N trichloracetic acid and then centrifuged. Control experiments were made with tissue heated in a boiling water bath for 10 min. All experiments were made in duplicate.

Incubation with gastro-intestinal contents. The GI tract of the rat was removed from the abdomen as rapidly as possible and divided into stomach, duodenum-ileum, and caecum. The colon and rectum were discarded. The content of each segment was washed into Tris–KCl buffer (pH 7·4) to an approximate concentration of 20 per cent suspensions and then centrifuged at 1000 *g*. Each supernatant was then diluted with an equal volume of buffer. Aliquots from the final suspensions were then used in the incubation mixtures. Controls were made with gastro-intestinal content heated to destroy the microflora. The conditions of incubation were as those described for other tissues.

Assay of nicotine metabolites and determination of microsomal protein and P-450. Nicotine and its metabolites were determined by the method of Beckett *et al.* [6]. Microsomal protein was determined by the procedure of Lowry *et al.* [7], as modified by Miller [8]. P-450 was assayed by the method of Omura and Sato [9].

Table 1. Tissue distribution of *N*-oxide reductase activity in the rat.*

Tissue	(−)-Nicotine formed†	Relative activity
Liver	40·49 ± 0·5	100·0
Small intestine (whole) + stomach	16·07 ± 0·6	39·7
Lung	2·90 ± 0·2	7·2
Spleen	1·57 ± 0·1	3·9
Kidney	3·28 ± 0·3	8·1
Heart	2·94 ± 0·2	7·3
Brain	0·0	0·0
Blood	0·0	0·0

* Activities are expressed as per cent of substrate used per 1 g wet wt of tissue per 30 min and given as the mean of duplicate values ± half the difference between these values. The incubation mixture contained the following in a final volume of 7 ml: 3 ml of boiled or unboiled tissue homogenate (1000 *g*) equivalent to 1 g of tissue (wet wt); and in μmoles; NADP, 3; G-6-P, 50; nicotinamide, 100; $MgCl_2$, 25; (−)-nicotine-1′-*N*-oxide (mixture of *trans*-and *cis*-diastereo isomers, 7:3), 3; G-6-P-dehydrogenase (1 mg/ml), 0·02 μl. Isotonic Tris buffer–KCl, pH 7·4, 4 ml. Incubation was made at 37° for 30 min with nitrogen (incubation in air abolished all activity) as the gas phase.

† Approximately 1·5 per cent reduction occurred with boiled tissues.

RESULTS AND DISCUSSION

Enzymatic reduction of *N*-oxide compounds has been exhaustively reviewed [1]. It would appear that the reaction is predominantly hepatic and is associated with the microsomes [3, 10–13]. Non-enzymatic reduction of *N*-oxide drugs has also been reported [14, 15].

Table 2. Reduction of (−)-nicotine-1′-*N*-oxide by rat intestinal segments and contents*

System†	(−)-Nicotine formed (nmoles/g/45 min)‡	Non-metabolized *N*-oxide (nmoles)	Total recovery§	Relative activity
10,000 *g* (Duod. to ileum)	1022 ± 12			100
10,000 *g* (Caecum)	312 ± 14			30·6
10,000 *g* (Stomach)	35 ± 9			3·4
Caecal content (1:10 dilution)	2750 ± 18	108·5 ± 5	95·6	100
Small intestinal content	847 ± 21	2033 ± 19	96·0	30·4
Stomach content	210 ± 16	2736 ± 14	98·2	7·64

* Flasks were incubated under N_2 at 37° for 45 min and contained in a final volume of 7 ml: 3 ml of 10,000 *g* supernatant equivalent to 1 g wet wt tissue or 3 ml of 500 *g* supernatant of gut content (1:10 dilution) in isotonic Tris–KCl buffer pH 7·4, and in μmoles: NADP[3], G-6-P, 50; nicotinamide 100; $MgCl_2$, 25; G-6-P-dehydrogenase (1 mg/ml), 0·02 μl; (−)-nicotine-1′-*N*-oxide, 3; controls were made with boiled or unboiled tissue but NADP was omitted.

† Preparations were made from pooled tissues from two rats.

‡ Mean of duplicate values ± half the difference between these values.

§ No cotinine was detected.

Enzymatic reduction of (−)-nicotine-1′-N-oxide by various rat tissues. The *N*-oxide was anaerobically reduced to different extents by a number of tissue homogenates (Table 1), the liver being the most active. The intestine had about 40 per cent of the activity of the liver; kidney, heart, lung and spleen had small but significant amounts of activity. Brain and blood showed no activity. About 1·5 per cent reduction occurred with boiled tissue preparations.

The data in Table 2 show that the most active segment of gut tissue was the small intestine (duodenum to ileum) and the stomach the least. The caecum displayed 30 per cent of the activity shown by the small intestine. These findings are compatible with the thesis that the mucosa of the small intestine is metabolically active whereas the stomach tissue has either very limited activity or none [16].

In contrast to gut tissue, the most active content of the gut segments was the caecal whereas the stomach contents again exhibited the lowest activity. This is not unexpected since, apart from the colon, the caecum is known to normally harbour the majority of microorganisms and the stomach, particularly after fasting, the least [17].

Intracellular localization of N-oxide reductase activity in rat liver and intestine. Preliminary experiments revealed that the nuclear fraction from either tissue possessed but a little reducing activity (2–3 per cent of the total activity). Table 3 shows that almost all of the activity was present in the 1000 *g* supernatant of both tissues and only a small fraction of this was lost after centrifugation at 10,000 *g*. Contrary to the common belief that the *N*-oxide reductase is of microsomal origin, the data show that the enzyme appears to have a dual locale. The present study indicates that the microsomal and soluble fractions both possess substantial activity, with the former fraction having nearly twice the activity of the latter. On the other hand, the mitochondria seem to contribute only 7–10 per cent of the total reductase activity, possibly due to microsomal contamination. It is also evident that the microsomal and soluble *N*-oxide reductases appear to require a reduced nucleotide for activity (Table 3, footnote †). The data further indicate that these enzymes are non-specific in their requirements for NADH and NADPH, although the latter produced a greater reduction (approximately two-fold more by the liver preparations and 10 per cent more by the intestinal fractions). This is also clear from the observation that washed microsomes (footnote †) were devoid of reducing capability but when supplemented with preformed NADH or with NADPH-generating system a profound activity was displayed. The present observation of the requirement for NADPH is at variance with earlier studies on the reduction of other *N*-oxides in a sense that the reported reductases are not NADPH-dependent [12, 13, 18]. However, Murray and Chaykin [10, 11] have purified and characterized a NADH-dependent *N*-oxide reductase which reduced nicotinamide-*N*-oxide. These authors asserted that the enzyme was a metalloflavoprotein. The results in Table 3 footnote * further indicate that the reduction of the substrate was entirely enzymatic since neither cofactor alone or boiled tissue in the presence or absence of cofactors could cause any significant reduction. Even unboiled preparations were not able to effect reduction in 45 sec, an interval believed to be sufficient for many non-enzymatic reductions to proceed. The data in Table 3 further show that the *N*-oxide reductase(s) activity of all cell fractions studied were totally abolished by heat. This is in harmony with previous reports that the *N*-oxide reductase(s) were entirely or predominantly heat labile, and inhibited by oxygen [12, 13, 18]. Addition of boiled soluble fraction to microsomes or boiled microsomes to unboiled soluble fraction restored the activity to near control levels (see also footnote †). It is of interest that approximately one-third the activity of the *N*-oxide reductase(s) was lost when dialyzed soluble fraction (dialysis against water for 24 hr) was combined with microsomes (Table 3, footnote ‖).

The foregoing observations suggest that the soluble fractions contain, in addition to other factors, a heat-stable unidentified activator(s). Also, a less effective activator appears to be present in microsomes. Because NADPH is provided by the NADPH-generating system which is located in the soluble fraction, these findings further indicate that the soluble fraction is a complete reducing system whereas the microsome is incomplete, lacking the NADPH-generating system and depends upon the soluble fraction for the supply of the required reduced nucleotide.

Preliminary kinetic studies. Incubation of varying concentrations of (−)-nicotine-1′-*N*-oxide (mixture of *trans*- and *cis*-diastereoisomers, 7:3) with hepatic or intestinal 10,000 *g* supernatants, revealed that little or no acitivity was produced nonenzymatically (Fig. 1, curves a and b). At a concentration of 3 μmoles the rate of reduction was almost linear for both systems (curves a and b). Furthermore, the activity of the intestinal preparation at this concentration appears to be about 60 per cent of that of the liver. On the other hand, higher concentrations did not increase substantially the reduction of the substrate by either preparation. This suggests that at 8 μmoles the enzyme was nearly saturated with substrate and that maximum activity was attained. Indeed, increasing the concentration beyond 8 μmoles did not cause further increases in reduction.

Figure 2 shows that the rate of reduction increased steadily and was also linear over a period of 1 hr of incubation. The activity rate of the hepatic preparation was approximately double that of the intestines at the end of incubation time. In additional experiments when the 1000 *g* supernatant was incubated with either *trans* or *cis* (−)-nicotine-1′-*N*-oxide under identical conditions (Fig. 3), the rate of reduction was also linear for a period of 1 hr as was the case with the mixture of the two isomers. The *trans* isomer was reduced twice as rapidly as the *cis*, however, similar

Table 3. Intracellular distribution of *N*-oxide reductase and the effect of NADH* and NADPH* on the activity of the enzyme in rat tissues†

System	Small intestine				Liver			
	NADH (−)-Nicotine formed (nmoles/g)‡	Relative activity	NADPH (−)-Nicotine formed (nmoles/g)‡	Relative activity	NADH (−)-Nicotine formed (nmoles/g)‡	Relative activity	NADPH (−)-Nicotine formed (nmoles/g)‡	Relative activity
1000 *g* Supernatant	1082·0 ± 15	100·0	1155·3 ± 11	100·0	1534·0 ± 18	100·0	2482·6 ± 16	100·0
10,000 *g* Supernatant	930·0 ± 8	86·1	1038·8 ± 10	90·0	1297·7 ± 12	84·6	2455·0 ± 19	98·8
Mitochondria	10·5 ± 3	0·98	11·95 ± 2	1·03	10·28 ± 3	0·67	18·74 ± 4	0·75
Microsomes	602·0 ± 7	55·5	1021·0 ± 12	88·4	1226·0 ± 11	83·3	2240·0 ± 7	90·1
Microsomes (boiled)§	ND¶		240·0 ± 3	2·1	ND¶		28·0 ± 2	1·2
Soluble fraction	540·0 ± 4	50·0	785·0 ± 5	67·6	1086·2 ± 9	70·9	1821·6 ± 8	73·4
Soluble fraction (boiled)§	ND¶		20·0 ± 5	1·7	ND¶		25·0 ± 3	2·0
Mitochondria + soluble fraction	580 ± 3	53·8	793·2 ± 4	68·4	1134·5 ± 9	72·0	1842·0 ± 17	74·0
Microsomes + soluble fraction‖	918·1 ± 9	84·9	995·0 ± 7	85·2	1234·0 ± 14	80·5	2315·5 ± 22	93·3
Microsomes + soluble fraction (boiled)	ND¶		828·0 ± 6	71·6	ND¶		2291·2 ± 13	92·3
Microsomes (boiled) + soluble fraction	ND¶		732·0 ± 8	63·4	ND¶		1703·0 ± 10	68·6

* Incubation with either preformed NADH or NADPH-generating system in the absence of tissue failed to cause any reduction of substrate. No reduction occurred with boiled or unboiled 1000 *g* supernatant in 45 sec.

† The incubation mixture contained the following in a final volume of 7 ml (isotonic Tris–KCl buffer, pH 7·4): 3 ml of tissue preparation equivalent to 1 g of tissue (wet wt)(prepared from pooled tissues of two rats) and in μmoles: NADP or NADH, 3; G-6-P, 50 Nicotinamide, 100; $MgCl_2$, 25; (−)-nicotine-1′-*N*-oxide (mixture of *trans*- and *cis*-diastereoisomers, 7:3), G-6-P-dehydrogenase (1 mg/ml), 0·02 μl. Incubation was at 37° for 45 min under N_2 (incubation in air abolished all activity). Control experiments were made with boiled tissue fractions or unboiled fractions in which the nucleotides were omitted.

‡ Mean values of two experiments over and above control levels ± half the difference between these values. Control values for boiled tissues were negligible and those for unboiled fractions in the absence of cofactors ranged from a very small amount for mitochondria and microsomes to about 25 for the soluble fraction and 75 to 120 for the other systems.

§ Tissue was heated in a boiling water bath for 10 min.

‖ Approximately one-third of the activity was abolished by dialysis of the soluble fraction.

¶ ND = not determined.

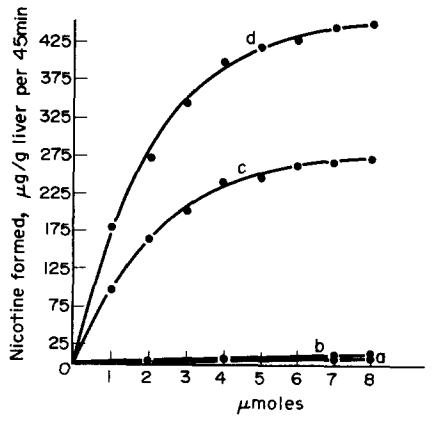

Fig. 1. Effect of varying the concentration of nicotine-1′-*N*-oxide on its enzymatic reduction by rat liver and intestinal homogenates. Typical of two such experiments: The incubation mixture contained in a final volume of 7 ml: 3 ml of boiled or unboiled tissue homogenate (10,000 *g*) equivalent to 1 g of tissue (wet wt) and in μmoles: NADP, 1; G-6-P, 50; nicotinamide, 100, $MgCl_2$,25; (−)-nicotine-1′-*N*-oxide (mixture of *trans*- and *cis*-diastereoisomers, 7:3), 1 to 8; G-6-P-dehydrogenase 0·02 μl. Incubation was made at 37° for 45 min with nitrogen as the gas phase. a = Liver homogenate; b = intestinal homogenate (whole small intestine); c = heated liver homogenate; d = heated intestinal homogenate. An increase of 16 per cent in the reduction of the *N*-oxide was caused by incubation with double concentration of tissue preparation under identical conditions.

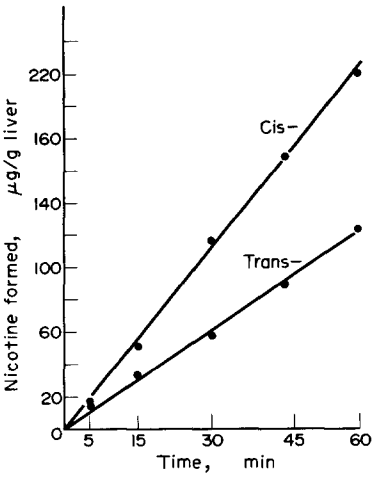

Fig. 3. Reduction of *cis*-and *trans*-diastereoisomers of (−)-nicotine-1′-*N*-oxide by rat liver. Typical of two such experiments. Incubation mixture and conditions of incubation were as described in Fig. 1. In this experiment however, 0·75 μmoles of the reinickate salt of either isomers dissolved in 0·5 ml of ethanol was used. In the presence of an equivalent quantity of reinickate to those present in the *cis*- and *trans*- salts of (−)-nicotine-1′-*N*-oxide (mixture of *cis*- and *trans*-7:3) reduction under identical conditions to nicotine proceeded to nearly the same extent as in its absence (130·5 and 136·0 mcg, respectively).

structural specificity was observed for the dextro and laevo-isomers of (−)-nicotine-1′-*N*-oxide with guinea-pig liver preparations [3].

Inhibitors of N-*oxide reductase.* SKF 525A, an established potent inhibitor for many drug metabolizing enzyme systems [10] diminished the reduction of (−)-nicotine-1′-*N*-oxide by the NADH- and NADPH-linked microsomal reductases by approximately 20–50 per cent (Table 4), but that of the soluble fraction by only 14 per cent. The blocking of activity of the soluble fraction by this agent is indeed note-worthy since SKF 525-A has not been shown to affect microsomal or other reductases apart from the nitro reductase of the soluble fraction from certain mammalian livers [20].

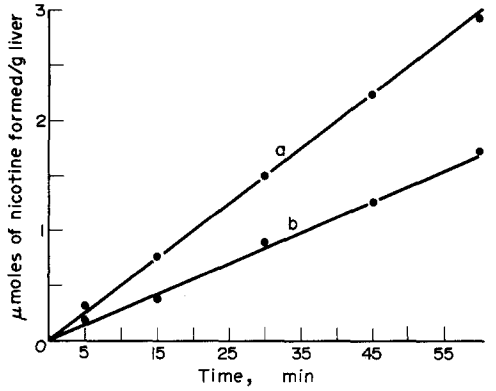

Fig. 2. Effect of duration of incubation on the enzymatic reduction of (−)-nicotine-1′-*N*-oxide by rat liver and intestine (10,000 *g* supernatant) Typical of two such experiments. The incubation mixture and conditions are as described in the legend of Fig. 1. Three μmoles of substrate were used in all incubations. a = liver homogenate; b = whole intestinal homogenate. Only a trace of reduction was observed by tissues in 45 sec in the absence of cofactors but not in 30 sec.

A more pronounced inhibition (approx. 90 per cent) of the two systems from both tissues was caused by incubation under CO (Table 4). This observation is in conflict with the reports that the *N*-oxide reductase activity with respect to certain *N*-oxide compounds are not affected by CO [12, 13]. The discrepancy could be attributed to either the types and specific properties of substrates used or to methodological differences. It is of interest that rat microsomal nitro and azo reductases were inhibited by CO [21, 22]. An extensive blocking by cyanide was also observed in the present study.

The foregoing inhibition experiments would suggest that at least the microsomal *N*-oxide reductase is a P-450-linked cytochrome. This speculation is compatible with the known inhibition of cytochromes by cyanide and the blocking effect of CO on a variety of NADPH-dependent systems through its binding with reduced microsomal cytochrome P-450. Cytochrome b_5-linked enzymes would not be affected by CO, however. Nonetheless, the present evidence does not preclude the possibility that reduction of (−)-nicotine-1′-*N*-oxide might have been mediated by enzymes other than non-cytochrome enzymes [23, 24].

Table 4. Effect of inhibitors on the *N*-oxide reductase activity in rat tissue preparations*

System†	Inhibitor added	Small intestine NADH (−)-Nicotine formed (nmoles/g)‡	Relative activity	Small intestine NADPH (−)-Nicotine formed (nmoles/g)‡	Relative activity	Liver NADH (−)-Nicotine formed (nmoles/g)‡	Relative activity	Liver NADPH (−)-Nicotine formed (nmoles/g)‡	Relative activity
Microsomes		473·6 ± 7	100	1098·0 ± 13	100	885·8 ± 10	100	2071·1 ± 14	100
	SKF 525A(10^{-3} M)	347·5 ± 3	73	853·0 ± 8	78	559·0 ± 8	63	1102·3 ± 7	53
	KCN(10^{-3} M)	39·8 ± 2	8	62·0 ± 5	6	51·4 ± 3	6	104·9 ± 11	5
	EDTA(10^{-2} M)	340·0 ± 6	72	815·7 ± 7	74	611·0 ± 11	68	1351·5 ± 9	65
	n-Prop-gallate (5×10^{-3} M)	394·0 ± 9	83	834·0 ± 6	76	753·0 ± 9	85	1698·0 ± 15	82
	CO	35·0 ± 4	7	21·4 ± 1	2	33·2 ± 4	4	63·4 ± 5	3
Soluble fraction		454·9 ± 9	100	853·2 ± 8	100	745·7 ± 12	100	1653·4 ± 18	100
	SKF 525A(10^{-3} M)	401·0 ± 5	88	749·0 ± 11	87	648·3 ± 10	87	1425·6 ± 16	86
	KCN(10^{-3} M)	52·7 ± 3	12	98·4 ± 4	9	84·0 ± 4	11	207·8 ± 7	13
	EDTA(10^{-2} M)	325·0 ± 5	71	590·0 ± 13	68	558·0 ± 8	75	1177·0 ± 9	70
	n-Prop-gallate (5×10^{-3} M)	239·0 ± 7	53	383·0 ± 7	45	373·0 ± 9	50	793·0 ± 10	48

* Flasks were incubated under N_2 at 37° for 45 min and contained in a final volume of 7 ml with isotonic Tris–KCl Buffer pH 7·4: 3 ml tissue preparations equivalent to 1 g wet wt and in μmoles: NADP, 3; G-6-P, 50; Nicotinamide, 100; $MgCl_2$, 25; (−)-nicotine-1′-*N*-oxide (mixture of *trans*- and *cis*-diastereoisomers, 7:3), 3; G-6-P dehydrogenase (1 mg/ml), 0·02 μl.

† Prepared from pooled tissues of two rats.

‡ Mean values of two experiments ± half the difference between these values. Total recoveries of nicotine formed and non-metabolized (−)-nicotine-1′-*N*-oxide ranged from 98 to 100 per cent.

The absence of P-450 from the intestinal microsomes would argue in favour of the presence in this fraction of an *N*-oxide reductase not linked to P-450. Despite inhibition by CO, this speculation is strengthened by the observation that the reductase activity was inhibited by cyanide and *n*-propyl gallate (Table 4), indicating that the enzyme could be a NADPH-dependent cytochrome-*c* reductase. In the present study inhibition of about 15–25 per cent of the microsomal reductase and 50 per cent of the intestinal enzyme by *n*-propyl gallate was noticed. It is to be recalled that *n*-propyl gallate is a known inhibitor of NADPH-dependent cytochrome-*c*-reductases [25]. Based on the same parameters it is reasonable to speculate that the (−)-nicotine-1′-*N*-oxide reductases of both the hepatic and soluble fractions could be NADPH-dependent cytochrome-*c* reductases or certain other metalloenzymes such as xanthine oxidase. The latter enzyme has been reported to reduce nicotinamide-*N*-oxide and is inhibited by cyanide [11] and CO[23].

Substantial blocking of the *N*-oxide reductase activity was also caused by high concentration of EDTA (Table 4). It is to be recalled that dialysis of the soluble fraction decreased its activity by one-third (Table 3, footnote §). When Mg^{2+} was excluded from the incubation milieu in preliminary experiments on microsomes not recorded in the table a substantial portion of the activity was lost (30 per cent). Thus, EDTA, at the concentration used, could have antagonized the reduction of the substrate by removing metal ions needed for optimum activity of the reductases.

Acceleration of N*-oxide reductase activity by flavins.* The enhancing action of flavins on the activity of the reductase(s) is evident from the data recorded in Table 5. Although no attempt was made to first dissociate the prosthetic group from the enzyme protein by mild acid treatment, the requirement for flavins was suggested by the profound increase in reduction of the substrate after addition of each of the flavins. Both the microsomal and soluble fraction enzymes were equally responsive to the flavins. Reduction by the hepatic and intestinal microsomes increased 2·5, 3 and 4-fold by FAD, FMN and riboflavin, respectively. A slightly greater enhancement was also observed with the soluble fractions. Thus it is possible that *N*-oxide reductases are actually flavoproteins with either FAD or FMN as their prosthetic groups. A NADH-dependent *N*-oxide reductase which was characterized by Murray and Chaykin [10, 11], as a flavoprotein has already been alluded to above. It is not clear however, why these reductases utilize riboflavin more efficiently than either FAD or FMN in view of the fact that this flavin has never been shown to act as a prosthetic group to any known enzyme although it serves as a precursor to flavin nucleotides. Nor is it clear how acceleration of enzyme activity occurred after the addition of excess flavins to an enzyme presumed to be an undissociated flavoprotein. Fouts and Brodie [20] postulated, as an explanation to similar observations on mammalian nitro-reductases, that the flavins can possibly act in the free form as artificial carriers between NADPH and the substrate. It is not unreasonable to extend the foregoing postulate to the present study as well. Undoubtedly further work is needed to confirm this hypothesis.

The effect of pretreatment of rats with phenobarbital on the N*-oxide reductase activity and the level of protein of the subcellular fractions as well as that of P-450.* Pretreatment of rats with phenobarbital (80 mg/kg daily for 4 consecutive days by i.p. administration) caused a 25–50 per cent increase in the reduction of (−)-nicotine-1′-*N*-oxide (Table 6). Except for the hepatic soluble fraction the concentration of nicotine formed was roughly proportionate to an increase in the level of the subcellular protein. Likewise, the reduction of (−)-

Table 5. Enhancement of *N*-oxide reductase activity by flavins* in rat tissue preparations†

System‡	Addition (1×10^{-3} M)	Intestine (−)-Nicotine formed (nmoles/g)§	Intestine Relative activity	Liver (−)-Nicotine formed (nmoles/g)§	Liver Relative activity
Microsomes		567 ± 6	100	1613 ± 8	100
	FAD	1701 ± 9	249	4153 ± 21	258
	FMN	1965 ± 11	294	4940 ± 15	307
	Riboflavin	2565 ± 8	387	6830 ± 23	423
Soluble fraction		415 ± 7	100	1286 ± 11	100
	FAD	1346 ± 10	324	4589 ± 18	355
	FMN	1467 ± 15	353	5520 ± 25	428
	Riboflavin	1675 ± 17	403	5750 ± 22	442

* No reduction by the flavins (10^{-3} M) could be detected in the absence of tissue preparations.

† The incubation mixture contained the following in a final volume of 7 ml (isotonic Tris–KCl buffer pH 7·4): 3 ml tissue preparation equivalent to 1 g wet wt and in μmoles: NADP, 3; G-6-P, 50; nicotinamide 100; $MgCl_2$, 25; (−)-nicotine-1′-*N*-oxide (mixture of *trans*- and *cis*-diastereoisomers, 7:3), 9; G-6-P-dehydrogenase (1 mg/ml) 0·02 μl. Incubation was at 37° for 1 hr under N_2. Control experiments were made with boiled tissue fractions or unboiled fractions but NADP was omitted.

‡ Prepared from pooled tissues obtained from two rats.

§ Mean of duplicate values ± half the difference between these values.

Table 6. Effect of pre-treatment with phenobarbital* on the *N*-oxide reductase activity of liver and intestine, and the level of hepatic microsomal protein and P-450 in rat†

	Small intestine				Liver			
	Microsomes		Soluble fraction		Microsomes		Soluble fraction	
Substance formed	Treated	Untreated‡	Treated	Untreated‡	Treated	Untreated‡	Treated	Untreated‡
(−)-Nicotine (nmoles/g)§	2299 ± 13	1021·0 ± 12	1340 ± 22	785 ± 5	3950 ± 7	2240 ± 7	2477 ± 25	1821 ± 7
Protein of tissue fraction (mg/g tissue)	22·5	10·5	36·8	17·0	35·5 ± 0·3	19·2 ± 0·2	42·8	41·4
P-450 (nmoles/mg protein)	0·0	0·0	—	—	1·3	0·66	—	—
Relative activity	100	44·4	100	58·5	100	56·8	100	75·0

* Rats were injected intraperitoneally with phenobarbital sodium (80 mg/kg body wt) dissolved in 0·5 ml sterile saline.

† The incubation mixture contained the following in a final volume of 7 ml (isotonic Tris–KCl buffer pH 7·4): 3 ml tissue preparation equivalent to 1 g wet wt and in μmoles: NADP, 3; G-6-P, 50; nicotinamide, 100; $MgCl_2$, 25; (−)-nicotine-1′-*N*-oxide (mixture of *trans*- and *cis*-diastereoisomers, 7:3), G-6-P-dehydrogenase (1 mg/ml), 0·02 μl. Incubation was under N_2 at 37° for 45 min. Controls were made with boiled tissue fractions or unboiled fractions but NADP was omitted.

‡ Copied from Table 2 for easy comparison.

§ Mean value of two experiments on separate tissues from two rats ± half the difference of these values.

nicotine-1′-*N*-oxide by the treated and untreated rats appears to approximately parallel the increase in P-450. The results in Table 6 are not only compatible with the earlier contention in that at least a portion of the hepatic *N*-oxide reductase activity is probably linked to P-450 but other NADPH-dependent enzymes could also be involved. This contention is strengthened by the fact that no P-450 was detected in the microsomes from the small intestines. The observation that the extent of reduction by the hepatic soluble fraction was not commensurate with the level of protein in this fraction is not clear at present.

Although the evidence obtained in the present study is not conclusive, it is nonetheless strongly suggestive that the anaerobically functioning (−)-nicotine-1′-*N*-oxide reductases could be flavoprotein NADPH-dependent reductases which are different in some respects from nitro reductases or other reductases. Moreover, reduction of the *N*-oxide does not seem to be the reversal of *N*-oxide formation from (−)-nicotine since apart from its requirement for oxygen the oxidizing system was not inhibited by KCN or SKF 525-A [26] as were the *N*-oxide reductases. However, the fact that the substantial reduction observed in preliminary experiments (40–50 per cent) occurred in perfused livers would suggest that an as yet unidentified reductase system may have also functioned under aerobic conditions which could be the same oxidizing system which was responsible for the formation of (−)-nicotine-1′-*N*-oxide from (−)-nicotine. It is noteworthy that a profound reduction of certain other *N*-oxide drugs in perfused livers of some animal species has also been reported. Conversely, the alleged unidentified reductase system could have been the same anaerobic reductase system which would require structural integrity in order to manifest its activity. This could explain why reduction did not occur aerobically after cell disruption by homogenization but proceeded in perfused livers. A partial support to these contentions was obtained from the *in vivo* experiments after i.p. administration of the *N*-oxide which revealed considerable reduction of the drug following this route of administration. These experiments are the subject of a forthcoming communication.

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