Biochemical Pharmacology, Vol. 20, pp. 407–415. Pergamon Press, 1971. Printed in Great Britain

ENZYMIC OXIDATION OF (−)-NICOTINE BY GUINEA-PIG TISSUES *IN VITRO*

J. BOOTH and E. BOYLAND

Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London, S.W.3

(*Received* 27 *April* 1970; *accepted* 23 *July* 1970)

Abstract—(−)-Nicotine is oxidized *in vitro* by NADPH and oxygen dependent mixed function oxidases of guinea-pig tissues to two optically-active stereoisomers of nicotine-1′-oxide and to cotinine when the soluble fraction is also present. Of the tissues examined liver is the most active in synthesizing these three metabolites although there is some activity in lung and kidney but little or none in brain, heart, spleen or blood. The ratios of the amounts of the three metabolites vary with different tissues. The substrate concentration affects the rate of formation of these oxidation products differently, relatively large amounts of *laevo*-nicotine-1′-oxide being synthesized at the lower (−)-nicotine concentrations. None of the three metabolites is metabolized further when incubations are in air, but under anaerobic conditions nicotine-1′-oxides are enzymically reduced to nicotine at different rates. With initial substrate concentration of 0·4 mM, *laevo*-nicotine-1′-oxide is reduced six times as rapidly as the *dextro*-rotatory isomer.

THE SMOKING of tobacco leads to the absorption, metabolism and urinary excretion of nicotine and some of its metabolites.[1] A review of the distribution and fate of nicotine shows that this alkaloid is metabolized by a variety of reactions,[2] but the present work is concerned only with enzymic oxidations.

Previous investigations have shown that rabbit liver preparations catalyse the oxidation of (−)-nicotine *in vitro*, to cotinine[3] and nicotine-1′-oxide[4] in the presence of NADPH and oxygen. The enzyme systems concerned in nicotine-1′-oxide formation show stereospecificity since two stereoisomers of this metabolite are synthesized by rabbit, hamster, mouse and guinea-pig liver and by guinea-pig lung, the relative amounts of the isomers depending on the species.[5] In *in vivo* experiments the two stereoisomers of nicotine-1′-oxide have been identified in the liver of the cat after administration of [^{14}C]nicotine[6] and in the urine of cigarette smokers.[5]

The existence of two stereoisomers of nicotine-1′-oxide is due to different arrangements of groups at the quaternary nitrogen atom of the pyrrolidine ring, the methyl and pyridyl groups being *cis* to each other in one isomer and *trans* in the other.[7] Although these isomers are not enantiomorphs one is *dextro*- and the other *laevo*-rotatory. Hence they are referred to as *dextro*- and *laevo*-nicotine-1′-oxides as previously.[5]

The present paper describes some investigations of the enzyme systems in guinea-pig tissues which are concerned in the enzymic oxidations of (−)-nicotine to cotinine and *dextro*- and *laevo*-nicotine-1′-oxides.

MATERIALS

(−)-Nicotine was purified and (−)-cotinine and nicotine-1′-oxide were prepared as described previously.[5] Nicotine-1′-oxide is deliquescent, and the most suitable crystalline derivative for use as a standard reference compound was the dipicrate which was

prepared by the method of Pinner and Wolffenstein.[8] Solutions of separate isomers were obtained by preparative paper chromatography of the monoreineckates.[5] Charcoal (decolourizing) was purchased from May & Baker Ltd., Dagenham, England and purified and suspended in water (10 g/100 ml) as described by Smith.[9] Iodoplatinate reagent[10] was used for the location of nicotine and nicotine-1′-oxide isomers on paper and thin-layer chromatograms.

METHODS

Chromatography. (−)-Nicotine and (−)-cotinine were estimated by gas–liquid chromatography using lignocaine and chlorphentermine respectively as internal standards.[11] Chromatograms were developed on Whatman 3MM paper with *n*-butanol–*n*-propanol–2N NH_4OH (2:1:1) by the descending technique. Thin-layer chromatography plates were coated with 0·25 mm Silica gel $HF_{254\,+\,366}$, (E. Merck A. G., Darmstadt, Germany) and activated at 100° for 1 hr. Ascending chromatograms were developed with acetone–benzene–ethanol–ammonia (Sp. gr. 0·88) (40:50:5:3).

Tissue preparations. For standard incubations fresh tissue from male guinea-pigs was homogenized in 3 vol. cold KCl (1·15% w/v) using an Ultra-Turrax homogenizer. The homogenates were centrifuged at 5° in an Angle 50 centrifuge (Measuring and Scientific Equipment Ltd., London) for 20 min at 2650 g_{max}. The supernatant phase was used for incubations and is referred to as the tissue supernatant.

For sub-cellular fractionation of liver homogenates, livers from male guinea-pigs were homogenized in 3 vol. cold 0·25 M sucrose with a Potter–Elvehjem type homogenizer. The homogenates were centrifuged at 700 $g_{av.}$ for 10 min in the Angle 50 centrifuge. The sediment is referred to as the nuclear fraction and the supernatant phase was centrifuged at 9000 $g_{av.}$ for 20 min in a Measuring and Scientific Equipment Ltd. Automatic Superspeed 65 preparative ultra-centrifuge, using the 8 × 25 ml angle Rotor. This sediment is referred to as the mitochondrial fraction and the supernatant phase was centrifuged at 140,000 $g_{av.}$ for 1 hr in the same centrifuge using the 10 × 10 ml Angle Rotor. The sediment is referred to as the microsomal fraction and the supernatant phase as the soluble fraction. All sediments were suspended in original volumes of 0·25 M sucrose and the appropriate centrifugation procedure repeated. They were then resuspended in 0·1 M phosphate buffer pH 7·5, so that 1 ml suspension was equivalent to 250 mg liver.

Determination of enzyme activity. (−)-Nicotine was incubated with fortified guinea-pig tissues and the synthesized cotinine extracted into dichloromethane. Nicotine-1′-oxides, which remained in the aqueous phase, were separated by paper chromatography and reduced to nicotine. Cotinine and nicotine were estimated by gas–liquid chromatography. Thus the amounts of the three metabolites could be determined in the same reaction mixture. Standard incubation mixtures (6 ml) contained sodium phosphate buffer pH 7·5 (300 μmole); a NADPH generating system consisting of NADP (1 μmole), glucose 6-phosphate (50 μmoles) and glucose 6-phosphate dehydrogenase (5 μg); nicotinamide (120 μmoles); (−)-nicotine (36 μmoles) and male guinea-pig tissue supernatant (1 ml). Incubations were for 30 min at 37° in air. Any changes from the standard mixtures are reported in the tables and figures. The reactions were started by the addition of (−)-nicotine after 10 min preincubation and stopped by the addi-

tion of 1 ml trichloroacetic acid (36% w/v) and 0·25–1·0 ml of lignocaine solution (100 μg/ml). After removal of the protein by centrifugation, the supernatant was treated with 1 ml of 5 M NaOH and extracted with three 10 ml portions of dichloromethane. The first two extracts were combined, dried over sodium sulphate, filtered and concentrated to approximately 0·2 ml on a rotary evaporator for cotinine estimations.

After extraction with dichloromethane, the aqueous phase was treated with glacial acetic acid (1 ml) and charcoal (300 mg). The charcoal was removed by filtration with suction, washed on the funnel with 20 ml water and nicotine-1′-oxides were eluted with 15 ml 3% (v/v) ammonia in methanol. The eluate was evaporated to dryness on a rotary evaporator, the residue dissolved in 0·25 ml methanol and 0·2 or 0·1 ml of the solution applied to 3 MM chromatography paper as a 2·5 cm band. After development of the chromatograms the nicotine-1′-oxides were located as dark bands when viewed under ultraviolet light or as black bands when sprayed with iodoplatinate reagent. Appropriate areas of paper were cut out and the oxides reduced to nicotine with 5 ml 0·2 N titanous chloride in the presence of 0·5 ml 7·3 M ammonium thiocyanate.[12] After 2 hr the reduction was stopped by the addition of 5 M NaOH (3 ml) and 0·25–1 ml solution of chlorphentermine (100 μg/ml) added. Solutions were filtered and the filtrates extracted twice with 10 ml ether. Ether extracts were combined, dried over sodium sulphate and evaporated to approximately 0·2 ml for nicotine determinations. Standard curves were obtained by adding known amounts of cotinine and nicotine-1′-oxides to complete unincubated reaction mixtures. Controls consisted of complete mixtures, with substrate added at the end of the incubation or with tissue omitted.

In anaerobic incubations with nicotine-1′-oxides as substrates, reactions were started by addition of substrate after 10 min preincubation in an atmosphere of nitrogen. They were stopped by the addition of 1 ml trichloroacetic acid (36% w/v) and 1 ml chlorphentermine solution (100 μg) was added. After removal of the protein by centrifugation, the supernatant phase was treated with 5 M NaOH (1 ml) and extracted twice with 10 ml ether. Ether extracts were treated as before, using known amounts of nicotine added to complete unincubated reaction mixtures as standards and complete mixtures with substrates added at the end of the incubation as controls.

RESULTS

Factors affecting the enzymic oxidation of (−)*-nicotine by guinea-pig liver.* With all the other constituents as described for standard incubation mixtures, experiments with sodium phosphate, sodium pyrophosphate and tris-HCl buffer solutions at various pH values showed that sodium phosphate was most satisfactory and that maximum activities occurred between pH values of 7·7 and 8·3. Hence sodium phosphate (final concn 0·05 M) pH 7·5 which maintained complete reaction mixtures at pH 7·8 was used throughout. The rate of formation of cotinine and *dextro-* and *laevo*-nicotine-1′-oxides was proportional to incubation times up to 45 min using 300 mg liver and to liver concentration up to 450 mg liver using 30 min incubation times. Table 1 shows that the NADPH generating system is essential (Expts. 1 and 2) and that sufficient is present in standard mixtures for maximum activity (Expt. 3). The NADPH generating system can be replaced by NADPH (Expt. 4) but not by NADH (Expt. 5) and omission of nicotinamide results in reduced activity (Expt. 6). Oxygen is also essential for these

TABLE 1. FACTORS AFFECTING THE ENZYMIC OXIDATION OF (−)-NICOTINE

Expt.		Metabolites formed (% Standard incubation)		
		Cotinine	*Dextro*-nicotine-1′-oxide	*Laevo*-nicotine-1′-oxide
1	Standard incubation	100	100	100
2	NADPH generating system omitted	22	9	3
3	NADPH generating system increased two fold	85	108	103
4	NADPH generating system replaced by NADPH (2 μmoles)	71	89	88
5	NADPH generating system replaced by NADH (2 μmoles)	38	9	0
6	Nicotinamide omitted	98	64	50
7	Incubations in N_2 atmosphere	0	0	0
8	Liver supernatant heated (5 min at 100°)	0	0	0
9	Standard incubation + 1 ml soluble liver fraction	100	—	—

Reaction mixtures using 1 ml liver supernatant (300 mg liver) were incubated in air for 30 min at 37°. The values shown are typical of three similar experiments.

oxidations since when incubations are carried out in an atmosphere of nitrogen instead of air no metabolites were formed (Expt. 7) and the enzymic nature of the reaction is demonstrated by Expt. 8 which shows there is no oxidation when the liver is heated (5 min at 100°) before incubation. The biosynthesis of cotinine probably involves oxidation of nicotine to 5′-hydroxynicotine by the microsomal fraction followed by dehydrogenation of the 5′-hydroxynicotine to cotinine by the soluble fraction.[3] Addition of 1 ml soluble fraction to complete incubation mixtures did not increase the yield of cotinine (Expt. 9) showing that the dehydrogenation was not the rate limiting step in cotinine synthesis. The effect of substrate concentration on the rate of formation of the enzymic oxidation products of (−)-nicotine is shown in Fig. 1. The ratio of the three metabolites varied with substrate concentration and nicotine appears to have the greatest affinity for the enzyme that synthesizes *laevo*-nicotine-1′-oxide. Thus the lower (−)-nicotine concentrations favour the formation of relatively large amounts of *laevo*-nicotine-1′-oxide compared with the amounts of the other two metabolites.

Subcellular distribution of nicotine oxidizing enzymes. Table 2 shows that the enzyme systems which oxidize (−)-nicotine to both isomers of nicotine-1′-oxide are located in the microsomal fraction of guinea-pig liver but that the activity is increased by the addition of the soluble fraction. The oxidation of (−)-nicotine to cotinine, however, requires both the microsomal and the soluble fraction as reported previously.[3]

Enzymic oxidation of (−)-nicotine by various guinea-pig tissues. Estimations of the abilities of various guinea-pig tissues to oxidize (−)-nicotine (Table 3) show that liver is more active in synthesizing cotinine and both isomers of nicotine-1′-oxide than any of the other tissues examined, although kidney and lung also synthesize appreciable amounts of the three metabolites. The relative amounts of nicotine-1′-oxides formed by the active tissues vary, the main difference being the large preponderance of the *laevo*- over the *dextro*-rotatory isomer which is synthesized by lung tissue. Blood, brain, heart and spleen show little or no activity.

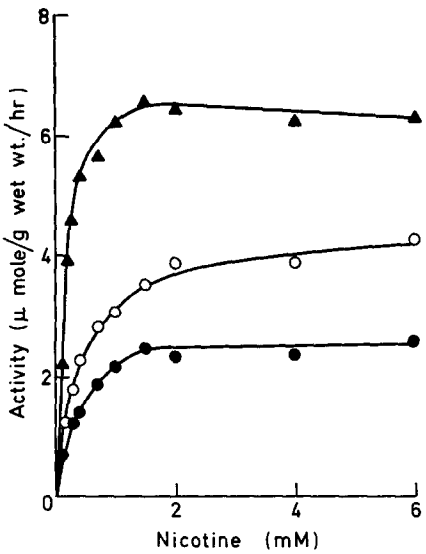

FIG. 1. Effect of substrate concentration on the enzymic oxidation of (−)-nicotine to cotinine (●) and *dextro*-(○) and *laevo*-rotatory (▲) nicotine-1′-oxide by guinea-pig liver. Standard incubation mixtures using 1 ml liver supernatant (300 mg liver) were incubated in air for 30 min at 37°. The data are typical of four similar experiments.

Further metabolism of the enzymic oxidation products of (−)-*nicotine.* To determine if any of the oxidation products of (−)-nicotine were further metabolized, liver supernatants were incubated with concentrations of cotinine (0·1 mM) or nicotine-1′-oxides (each isomer 0·2 mM) which were similar to those present at the end of a typical incubation of liver supernatant with (−)-nicotine. Standard reaction mixtures were pre-incubated for 10 min in air or N_2, the substrates added and the amounts of each estimated at zero time and after incubation. When incubations were for 30 min in air 95 per cent cotinine, 98 per cent *dextro*-nicotine-1′-oxide and 94 per cent *laevo*-nicotine-1′-oxide were recovered indicating that no further metabolism had occurred and that neither of the nicotine-1′-oxides was converted to the other isomer. However,

TABLE 2. SUBCELLULAR DISTRIBUTION OF (−)-NICOTINE OXIDIZING ENZYMES

	Metabolites formed (% of homogenate)		
Cell fraction	Cotinine	*Dextro*-nicotine-1′-oxide	*Laevo*-nicotine-1′-oxide
Homogenate	100	100	100
Nuclear	6	2	0
Mitochondrial	6	4	0
Microsomal	9	81	79
Soluble	0	0	0
Microsomal + soluble	95	88	92

Fractions of liver homogenate corresponding to 250 mg liver were incubated in standard mixtures for 30 min at 37° in air. Centrifugation procedures are described in the Methods section. The values shown are typical of three similar experiments.

TABLE 3. ENZYMIC OXIDATION OF (−)-NICOTINE BY VARIOUS GUINEA-PIG TISSUES

Tissue	Metabolites formed (nmole/g wet wt./hr*)		
	Cotinine	*Dextro*-nicotine-1′-oxide	*Laevo*-nicotine-1′-oxide
Liver	2654 ± 242	4478 ± 198	6368 ± 298
Kidney	203 ± 58	249 ± 30	447 ± 40
Lung	198 ± 53	60 ± 6	288 ± 55
Brain	<20	<25	<25
Heart	<20	<25	<25
Spleen	<20	<25	<25
Blood	<20	<25	<25

* Mean of four experiments ± S.E. Standard incubation mixtures using 1 ml tissue supernatant or 1 ml blood haemolysate (blood haemolysed in 3 vol. water) were incubated for 30 min at 37° in air.

when incubations were in N_2, 96 per cent cotinine and 92 per cent *dextro*-nicotine-1′-oxide were recovered but only 68 per cent *laevo*-nicotine-1′-oxide remained after incubation. Further investigations of reaction products which were formed when liver supernatants were incubated with nicotine-1′-oxides under anaerobic conditions revealed the presence of nicotine. Nicotine was identified by thin-layer chromatography as described in the methods section. Both (−)-nicotine and the product from incubation mixtures had an R_f value of 0·58 and formed a black complex with the iodoplatinate reagent. Furthermore, the metabolite behaved in an identical manner to (−)-nicotine when subjected to gas–liquid chromatography under the conditions used for nicotine determinations.[11] In further experiments liver supernatant in standard

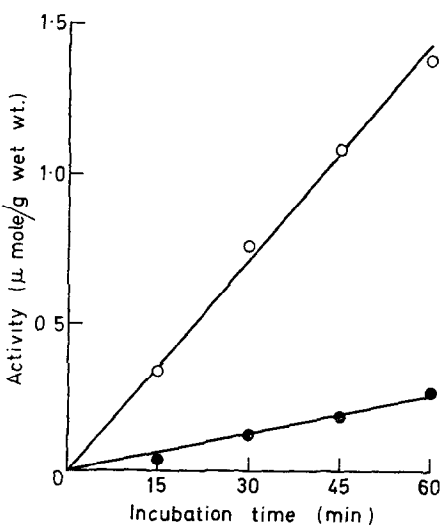

FIG. 2. Enzymic reduction of nicotine-1′-oxides to nicotine. Liver supernatant was incubated in standard mixtures with 0·4 mM *dextro*-(●) or *laevo*-rotatory (○) nicotine-1′-oxide at 37° in N_2. The data are typical of three similar experiments.

mixtures was incubated under N_2 with either *dextro*- (0·4 mM) or *laevo*-nicotine-1′-oxide (0·4 mM) and the amount of nicotine formed after various incubation times determined. Figure 2 shows that the rate of reduction of each isomer was linear for 1 hr but that, under these conditions, *laevo*-nicotine-1′-oxide was reduced six times as rapidly as the *dextro*-rotatory isomer. No reduction occurred when the liver was heated (100° for 5 min) before the incubations.

DISCUSSION

Microsomal mixed function oxidases which require NADPH and oxygen catalyze the metabolism of many drugs by a variety of reactions. The oxidation of (−)-nicotine to two stereoisomers of nicotine-1′-oxide provides a further example of a foreign compound that is metabolized by these enzyme systems, since all the activity of a guinea-pig liver homogenate has been located in the microsomal fraction and *N*-oxidation does not occur unless NADPH and oxygen are also present. A mixed function oxidase which catalyses the NADPH- and oxygen-dependent *N*-oxidation of a variety of amines has been purified and concentrated from pork liver microsomes.[13] In most of the substrates which are rapidly oxidized by the purified enzyme the nitrogen atom occurs in an alkyl side chain as in chlorpromazine. However, the oxidation of ring nitrogen is also catalyzed since the *N*-oxides of morphine and scopolamine have been identified as reaction products in experiments using the purified enzyme.[13] Nicotine was also shown to be a substrate for the purified enzyme by measurements of substrate-dependent NADPH oxidation.[13] The oxidation products were not identified but it would be of interest to determine if one or both isomers of nicotine-1′-oxide were synthesized by the purified enzyme.

The capacity of various tissues to metabolize nicotine has been studied with tissue slices from mouse[14,15] and from rabbit, cat and dog.[15] In all these species nicotine was metabolized by liver, kidney and lung, with liver showing the greatest activity and brain no activity. Similar results were obtained in the present work with guinea-pig tissues. Liver was the most active of the tissues examined in converting nicotine into cotinine and both isomers of nicotine-1′-oxide, but kidney and lung were also able to form all three metabolites. On the other hand, heart, spleen, brain and blood showed little or no activity. In all the active tissues more *laevo*- than *dextro*-nicotine-1′-oxide was formed but the ratio of *laevo*- to *dextro*-synthesized by lung tissue was particularly high. Results obtained by determining [^{14}C]nicotine and [^{14}C]cotinine in various tissues of the cat during the 2 hr following intravenous injections of 40 μg of [^{14}C]nicotine are of particular interest with respect to lung and suggest that this tissue concentrates nicotine metabolites.[6] Although all tissues showed a maximum [^{14}C]nicotine concentration 5 min after injection the proportion of radioactivity representing [^{14}C]nicotine itself was lower in lung than liver, kidney or brain. Since only a small part of the remainder was due to cotinine the relatively large amounts of radioactivity due to nicotine metabolites found in lung may be partly due to nicotine-1′-oxide formation by this tissue. Furthermore, in brain, which shows little or no metabolizing activity, 95·3 per cent of the radioactivity was due to [^{14}C]nicotine.[6] On the other hand, these differences may be due to variations in the capacities of the tissues to bind with nicotine and its metabolites[6] which are transported to the various sites after formation in the liver.

The accuracy of K_m determinations using crude enzyme preparations are open to criticism since several rate limiting steps are probably involved in the overall reactions catalyzed by mixed function oxidases. However, changes in (—)-nicotine concentration affected the rate of formation of cotinine and *dextro-* and *laevo*-nicotine-1′-oxides differently, and hence the ratio of the three oxidation products was dependent on substrate concentration. This suggests that (—)-nicotine binds to three sites on the endoplasmic reticulum and has the greatest affinity for the enzyme that synthesizes *laevo*-nicotine-1′-oxide since this is the most rapidly formed metabolite at low nicotine concentrations. The hypothesis that different *N*-oxidases are concerned in the formation of *dextro-* and *laevo*-nicotine-1′-oxides is supported by the different relative amounts of the two isomers which are synthesized by the livers of various species[5] and by different guinea-pig tissues *in vitro*. Under the *in vitro* conditions the enzymes were saturated with both (—)-nicotine and NADPH. The concentration of nicotine which would be available for oxidation after absorption by the smoking of tobacco would be very much lower and would therefore favour the synthesis of relatively more of the *laevo*-isomer of nicotine-1′-oxide. Hence the finding that it is *dextro*-nicotine-1′-oxide that predominates in the urine of cigarette smokers[5] is of interest. Although it is possible that, unlike other species studied, man synthesizes more of the *dextro*-rotatory isomer, another possibility is that the *laevo*-rotatory isomer undergoes further reaction *in vivo*, either by binding with cellular constituents or by further metabolism. One such reaction may be the preferential reduction of this isomer to nicotine, since under anaerobic conditions and with substrate concentration 0·4 mM, *laevo*-nicotine-1′-oxide is reduced six times as rapidly as the *dextro*-rotatory isomer by guinea-pig liver. Thus the oxidases that convert (—)-nicotine to nicotine-1′-oxides in the presence of oxygen show stereospecificity with respect to the product and the reductase(s) that catalyse the reverse reaction under anaerobic conditions exhibit stereospecificity with respect to substrate.

Both the oxidation of an amine to its *N*-oxide and the reduction of the *N*-oxide back to the parent compound by the same mammalian system have been reported for other amines such as chlorcyclizine,[16] chlorpromazine,[17] imipramine[18] and tremorine.[19] The mechanism of enzymic *N*-oxide reduction is not clear and reports of this reaction with various substrates have been reviewed.[20] They show considerable discrepancies with respect to such properties as heat stability, intracellular distribution and cofactor requirements with different substrates. Nicotinamide-*N*-oxide is also synthesized and enzymically reduced in biological systems.[21] Experiments with ^{18}O, in the presence of xanthine oxidase, showed that oxygen could be transferred from this oxide to xanthine thus raising the possibility that *N*-oxides may be able to act as general biological oxygenating agents.[22] Similarly, in some NADPH-dependent microsomal oxidations dimethylaniline-*N*-oxide can replace oxygen.[23] Furthermore, the trimethylamine: trimethylamine-*N*-oxide ratio in urine has been suggested as an index of the oxidative processes in the body and, in one series of experiments, was 46 per cent lower in cancer patients than in normal controls.[24] It is thus possible that when nicotine is absorbed by smoking tobacco, an oxidation–reduction system is set up between nicotine and nicotine-1′-oxides which interferes with similar systems concerned in cell metabolism and thus plays a part in some of the pharmacological effects of nicotine.

Acknowledgements—We thank Mr. W. Down for excellent technical assistance. This investigation has been supported by grants to the Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, from the Medical Research Council and the British Empire Cancer Campaign for Research.

REFERENCES

1. E. R. Bowman, L. B. Turnbull and H. McKennis, Jr., *J. Pharmac. exp. Ther.* **127,** 92 (1959).
2. H. McKennis, Jr., in *Tobacco Alkaloids and Related Compounds: 4th Wenner-Gren Center International Symposium*, Vol. 4 (Ed. U. S. Von Euler), p. 53. Pergamon Press, Oxford (1965).
3. H. B. Hucker, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **129,** 94 (1960).
4. N. M. Papadopoulos, *Archs Biochem. Biophys.* **106,** 182 (1964).
5. J. Booth and E. Boyland, *Biochem. Pharmac.* **19,** 733 (1970).
6. D. M. Turner, *Biochem. J.* **115,** 889 (1969).
7. J. C. Craig, N. V. Mary, N. L. Goldman and L. Wolf, *J. Am. Chem. Soc.* **86,** 3866 (1964).
8. A. Pinner and R. Wolffenstein, *Ber. dtsch. chem. Ges.* **24,** 61 (1891).
9. I. Smith, in *Chromatographic and electrophoretic techniques*, Vol. 1 (Ed. I. Smith) p. 242, Pitman Press, Bath (1960).
10. I. Smith, in *Chromatographic and electrophoretic techniques*, Vol. 1 (Ed. I Smith) p. 396. Pitman Press, Bath (1960).
11. A. H. Beckett and E. J. Triggs, *Nature, Lond.* **211,** 1415 (1966).
12. R. T. Brooks and P. D. Sternglanz, *Analyt. Chem.* **31,** 561 (1959).
13. D. M. Ziegler, C. H. Mitchell and D. Jollow, in *Symposium on Microsomes and Drug Oxidations* (Eds. J. R. Gillette, A. H. Conway, G. J. Cosmides, R. W. Estabrook, J. R. Fouts and G. J. Mannering) p. 173. Academic Press, New York (1969).
14. E. Hannson, P. C. Hoffmann and C. G. Schmiterlöw, *Acta physiol. scand.* **61,** 380 (1964).
15. A. W. Miller, Jr. and P. S. Larson, *J. Pharmac. exp. Ther.* **109,** 218 (1953).
16. R. Kuntzman, A. Phillips, I. Tsai, A. Klutch and J. J. Burns, *J. Pharmac. exp. Ther.* **155,** 337 (1967).
17. P. F. Coccia and W. W. Westerfeld, *J. Pharmac. exp. Ther.* **157,** 446 (1967).
18. M. H. Bickel, H. J. Weder and H. Aebi, *Biochem. biophys. Res. Commun.* **33,** 1012 (1968).
19. A. K. Cho, A. S. Fairhurst, W. L. Haslett and D. J. Jenden, in *Biochemical and Neurophysiological Correlation of Centrally Acting Drugs* (Eds. E. Trabucchi, R. Paoletti and N. Canal), p. 75, 2nd Int. Pharmac. Meet., Prague, Pergamon Press, Oxford (1964).
20. M. H. Bickel, *Pharmac. Rev.* **21,** 325 (1969).
21. S. Chaykin and K. Bloch, *Biochim. biophys. Acta* **31,** 213 (1959).
22. K. N. Murray, J. G. Watson and S. Chaykin, *J. biol. Chem.* **241,** 4798 (1966).
23. H. L. Schmidt and M. Moeller, *Fed. Eur. biochem. Soc. Meet. Abstr.* **5,** 94 (1968).
24. K. Hinsberg and E. Gangl-Reuss, *Z. Krebsforsch.* **52,** 227 (1941).