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HYDROXYCOTININE METABOLISM IN RABBIT LIVER, LUNG AND KIDNEY TISSUE HOMOGENATES

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Hydroxycotinine metabolism has been investigated in rabbit liver, lung and kidney tissue homogenates, using NADP and NADPH. With NADPH, the liver was the most active organ in metabolizing hydroxycotinine yielding hydroxycotinine-N-oxide (16.3%) as the main metabolic product, along with only little cotinine. With NADP, the formation of cotinine was slightly higher in the kidney and lung tissue incubates than the corresponding formation of cotinine with NADPH. The qualitative identification of the substrate and the metabolic products was carried out by thin-layer chromatography, followed by their quantitative estimation by gas-liquid chromatography.

In the metabolism of nicotine both *in vitro* and *in vivo*, cotinine has been identified as the principal metabolite of nicotine (1, 2, 3, 4, 5). Hydroxycotinine has been shown to be a minor but important nicotine metabolite formed by further metabolism of cotinine by hydroxylation of the pyrrolidone ring. This latter metabolite has been identified *in vivo* in the smoker's urine (1, 6) and also in the urine of dogs (7, 8), rats (9, 10), mice (11), rhesus monkeys (12) and humans (13) treated with cotinine, and further in the urine of rabbits and dogs treated with (S)-cotinine-N-oxide (14). Several investigators have also demonstrated the presence of hydroxycotinine in tissue incubates of nicotine and cotinine (15, 16).

Researchers differ in opinion about the true structure of hydroxycotinine. *McKennis and co-workers* (6) proposed 3-hydroxycotinine as a satis-

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factory structure, while *Gorrod and Jenner* (17) reported the structure as 4-hydroxycotinine. *Dagne and Castagnoli* (12) established the structural configuration of the metabolic hydroxycotinine to be trans-1-methyl-3--(R)-hydroxy-5-(S)-pyridil-2-pyrrolidinone.

The metabolic conversion of toxic nicotine into less toxic metabolites, involves essentially a detoxification mechanism and the liver is the principal organ for such detoxification (18—20). Lung and kidney tissues also play a significant role (21—23).

Further metabolism of nicotine metabolites has not yet been extensively studied. Only the metabolism of cotinine and nicotine-l'-N-oxide has been investigated to some extent (7, 11, 13, 24). Further metabolism of hydroxycotinine has not yet been reported. The present paper deals with an *in vitro* metabolic study of hydroxycotinine in rabbit liver, lung and kidney tissue homogenates.

MATERIAL AND METHODS

Analytical grade nicotine was supplied by E. Merck, W. Germany. Cotinine was synthesized by the method of *McKennis and co-workers* (7). Hydroxycotinine was synthesized by a modified method of *Dagne and Castagnoli* (12) which included a two-stage extraction. The reaction mixture was evaporated to dryness and the residue was first extracted with warm acetone. The acetone extract was again evaporated and the solid residue was treated with dichloromethane. After evaporation, crystals of hydroxycotinine were obtained. Hydroxycotinine-N-oxide was synthesized by a similar method as reported for the preparation of nicotine-l'--N-oxide by *Taylor and Boyer* (25).

Healthy male albino rabbits, each weighing around 3.0 kg were killed and the liver, lung and kidney tissues were immediately excised and chilled in a refrigerator. A weighed quantity of each tissue was thoroughly homogenized with two volumes of ice cold 0.25M tris-KCl solution (pH 7.4) by means of a standard homogenizer. All the tissues were incubated in the form of whole tissue homogenates without any fractionation.

The test incubation mixtures contained NADPH (1.4 μmol), glucose-6--phosphate (10 μmol), nicotinamide (50 μmol), $MgCl_2$-$6H_2O$ (25 μmol), all dissolved together in water (1.0 ml); 0.1M phosphate buffer, pH 7.4 (2.0 ml); substrate (0.25 mmol) in water (1.0 ml) and tissue homogenate (2.0 ml equivalent to 5.0 g of the tissue), constituting a total incubation of 6.0 ml. The control mixtures with NADP as the principal co-factor contained 2.5 μmol of NADP, all others remaining exactly the same as above.

The open-mouth hard-glass test tubes containing the test and control incubation mixtures were placed in a thermostat and the incubations

were carried out at 37 ± 0.5 °C for 120 minutes with occasional shaking. At the end of incubation, the test tubes were rapidly cooled with a stream of cold water, and 0.5 ml of 6 M HCl was added to each incubate to terminate any further enzymatic reaction.

For extraction of incubates prepared with NADPH, the incubate was first divided into several smaller portions, and the following two extractions were repeatedly carried out:

The extract to analyse the unmetabolized substrate and metabolic cotinine was prepared by making a portion of the incubate alkaline with 1 M NH_4OH and extracting it repeatedly with 20 ml volumes of dichlormethane, followed by slow evaporation of the combinated extract to a small volume.

The extract for analysis of the metabolic hydroxycotinine-N-oxide was prepared by reducing a portion of the incubate with titanium chloride solution to convert the N-oxide, if any had formed, to hydroxycotinine, by extracting it with dichlormethane in an alkaline medium as above and evaporating it to a small volume.

For the extraction of incubates prepared with NADP, the incubate was first transferred from the test tube to a beaker along with 1.0 ml of water-washing. The pH was then adjusted to 9.0 with 2.0 ml of 1 M NH_4OH and about 4 volumes (25 ml) of chloroform-methanol (2:1) were added and stirred magnetically for 10 minutes.

The resulting partially emulsified mixture was then centrifuged at 3 000 rpm for 5 minutes. After that two clear liquid layers were observed, along with a compact protein mass. The upper methanol-water phase and the lower chloroform phase were separated, and the extracts were evaporated slowly on a warm water-bath to small volumes. Both control and test incubation mixtures were extracted in a similar manner.

The incubate extracts were analysed for cotinine, hydroxycotinine and hydroxycotinine-N-oxide (as hydroxycotinine) by means of a Perkin Elmer F-17 gas chromatograph, fitted with a 3% OV-17 Chromosorb G column (1 m x 5 mm) and a flame ionization detector, using nitrogen as the carrier gas. The following essential conditions were maintained in all experiments:

GC conditions for hydroxycotinine: column temperature = 80 °C, injector temperature = 100 °C, N_2 = 27 ml/min

GC conditions for cotinine: column temperature: 250 °C, injector temperature = 275 °C, N_2 = 55 ml/min

Hydrogen flow rate (25 ml/min) and air flow rate (250 ml/min) were always kept constant.

The compounds were quantitatively determined by comparing the well-defined peak heights (or peak areas) with those of standard solutions of authentic compounds prepared and analysed in identical conditions.

Table 1

Thin-layer chromatographic data

Solvent system	Sample tested	Development time (min)	R_f value
S—1	Cotinine	30	0.72
S—1	Hydroxycotinine	30	0.42
S—1	Hydroxycotinine-N-oxide	30	0.48
S—2	Cotinine	20	0.46
S—2	Hydroxycotinine	20	0.13
S—2	Hydroxycotinine-N-oxide	20	0.24

Apart from analysis by gas chromatography, the chloroform extracts and methanol-water extracts were also analysed qualitatively by thin-layer chromatography, using two suitable solvent systems designated as S-1 (conc. NH_4OH:acetone:benzene:ethanol = 5:40:30/25, v/v) and S-2 (toluene:acetone:methanol:conc. NH_4OH = 8:9:2:1, v/v). The S-2 system was best suited for clear distinction of cotinine, hydroxycotinine and hydroxycotinine-N-oxide. Table-1 shows the comparative R_f values of the three compounds obtained after spraying with Dragendorff's reagent.

RESULTS AND DISCUSSION

The metabolic data for the incubate extracts prepared with NADPH and NADP are separately tabulated in Table 2.

The amounts of recovered hydroxycotinine in experiments with NADP are the amounts estimated in chloroform extracts only. The methanol-water extracts also indicated the presence of some unmetabolized hydroxycotinine by thin-layer chromatography, but these extracts could not be quantitatively analysed for hydroxycotinine by gas chromatography because a very low column temperature was required for analysis.

The TLC analysis of the methanol-water extracts from the kidney and lung incubates also indicated clear bright spots of hydroxycotinine-N-oxide, but the presence of the N-oxide in the corresponding liver incubate extracts could not be clearly ascertained. However, the quantitative gas chromatographic analysis for hydroxycotinine-N-oxide from these methanol-water extracts could not be carried out.

Cotinine in chloroform extracts was estimated quantitatively by gas chromatographic analysis. The methanol-water extracts were also analysed for cotinine by the same method but none contained cotinine.

In the metabolic study with NADPH, the liver was found to be the most active organ, as the extent of hydroxycotinine metabolism in the

Table 2.

Hydroxycotinine metabolism in vitro. Figures represent means of six experiments. In experiments with NADP the amount of hydroxycotinine recovered is that from the chloroform extracts. The methanol-water extracts contained very small amount of hydroxycotinine. None of the methanol-water extracts contained any cotinine.

	Tissue homogenate		Hydroxycotinine			Cotinine		Hydroxycotinine-N-oxide	
			Applied	Recovered (mg)	Recovered (%)	Recovered (mg)	Recovered (%)	Recovered (mg)	Recovered (%)
With NADPH	Liver	test	48*	32.1	66.9	0.38	0.8	7.8	16.3
		control	48	36.0	75.0	0.54	1.1	4.5	9.4
	Kidney	test	48	43.5	90.6	0.09	0.2	—	—
		control	48	42.6	88.8	0.09	0.2	—	—
	Lung	test	48	42.5	88.5	0.06	0.1	—	—
		control	48	41.4	86.3	0.06	0.1	—	—
With NADP	Liver	test	96**	44.1	45.9	0.57	0.6	—	—
		control	96	64.4	67.1	0.35	0.4	—	—
	Kidney	test	48	19.7	41.0	1.24	2.6	—	—
		control	48	19.3	40.2	0.80	1.7	—	—
	Lung	test	48	17.1	35.6	1.60	3.3	—	—
		control	48	16.0	33.3	1.30	2.7	—	—

* 0.25 mM
** 0.50 mM

liver was much higher both with and without co-factors than in the lung and kidney tissues. The principal metabolite was hydroxycotinine-N--oxide, with a very little percentage of metabolic cotinine.

In the metabolic study using NADP, the metabolic pattern seems to have been different. The lower recovery of unmetabolized hydroxycotinine and the higher formation of metabolic cotinine in the lung and kidney tissues than in the liver, as well as the significant TLC detection of hydroxycotinine-N-oxide in the lung and kidney incubate extracts with practically no response of the N-oxide in the liver extracts indicate that the extent of metabolism in the liver was less prominent than in the lung and kidney tissue incubates.

Interestingly, the metabolic formation of cotinine, although quite insignificant in amount, is a peculiar characteristic of hydroxycotinine metabolism. The formation of hydroxycotinine-N-oxide apparently involves an oxidative mechanism.

Hydroxycotinine has a lower acute toxicity than nicotine but the experiments made on chromosomes indicated that it also has mutagenic properties (26). In this lies great importance of observing hydroxycotinine metabolism and studying the toxicity of its metabolites.

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Sažetak

METABOLIZAM HIDROKSIKOTININA U HOMOGENATIMA TKIVA JETRE, BUBREGA I PLUĆA ZECA

Uz prisustvo NADP i NADPH ispitivan je metabolizam hidroksikotinina u homogenatima jetre pluća i bubrega zeca. Identifikacija i određivanje supstrata i stvorenih metabolita, provedena je metodom hromatografije na tankom sloju i gasnom hromatografijom.

Posebno su istaknuti uslovi za hromatografiranje hidroksikotinina. Uz NADPH u homogenatima jetre metabolizam je bio usmjeren u prevođenju hidroksikotinina u hidroksikotinin-N-oksid. Uz NADP u homogenatima bubrega i pluća je uz hidroksikotinin-N-oksid nastajao i kotinin.

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