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NICOTINAMIDE NUCLEOTIDE ANALOGUES OF NICOTINE AND COTININE—ENZYMIC STUDIES*

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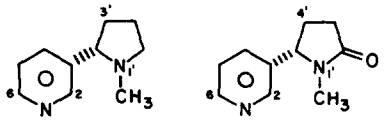
Abstract—Analogues of NAD and NADP were prepared from cotinine, the major metabolite of nicotine, through the NADase-catalyzed exchange reaction. These cotinine analogues and the corresponding nicotine analogues were hydrolyzed by snake venom nucleotide pyrophosphatase to produce the mononucleotides. The ribosides of nicotine and cotinine were prepared from the mononucleotides by hydrolysis with snake venom 5′-nucleotidase. The mononucleotide was the major product formed when (nicotine)AD or (cotinine)AD was incubated with a rabbit liver microsomal fraction. However, the ribosides were not detected when the mononucleotides were incubated with rabbit liver extract. If the nucleotide analogues are formed *in vivo*, the possibility exists that nicotine and cotinine mononucleotides could accumulate inside the cell.

The formation of NAD and NADP analogues of pyridine derivatives catalyzed by the NAD glycohydrolase (NADase, EC 3.2.2.5) exchange reaction has been the subject of extensive study [1]. The structurally modified products have different chemical properties and when substituted for NAD or NADP in enzyme–coenzyme interactions may exhibit entirely different activities [2]. The formation of these analogues within the intact animal has also been investigated in attempts to elucidate the toxicity, as well as the chemotherapeutic function, of some pyridine-related compounds [3–5]. Relatively little information is available about the metabolism of these analogues in mammalian tissues.

Nicotine (I), a 3-position substituted pyridine compound, is the major alkaloid in tobacco and is present in cigarettes in amounts ranging from 0.5 to 2.0 mg nicotine/cigarette [6]. The prime exposure to nicotine in the environment is through smoking; small quantities are also used in insecticides [7]. We have shown that in the presence of NADase, nicotine can be exchanged for the nicotinamide moiety of NAD and NADP [8, 9]. The NAD and NADP analogues of this alkaloid [(nicotine)AD and (nicotine)ADP‡] are inactive as coenzymes but can function as competitive inhibitors with respect to NAD and NADP with certain dehydrogenases [8, 9].

The preparation and characterization of the (nicotine)AD and (nicotine)ADP analogues were described in previous papers [8, 9]. Nicotine metabolites which are modified in the pyrrolidine ring but still retain an unaltered pyridine ring may also be exchanged for the nicotinamide moiety of NAD or NADP. Therefore, in attempts to assess their physiological importance in individuals who smoke tobacco, analogues formed from the metabolites of the alkaloid must also be considered. Nicotine is metabolized in a number of species to form cotinine (II) as the major product [10]. In human sera, this metabolite is far more abundant and longer lived than the parent compound Fig. 1 [11–13].



(−)-Nicotine (−)-Cotinine

I II

Fig. 1. Structural diagrams of nicotine and cotinine.

This paper describes the formation and characterization of NAD and NADP analogues of cotinine [(cotinine)AD and (cotinine)ADP] and the mononucleotides of nicotine [(nicotine)RP] and cotinine [(cotinine)RP]. The fate of these compounds when incubated in the presence of rabbit liver extract was also investigated. A preliminary report of this work has been presented [14].

MATERIALS AND METHODS

Chemicals and enzymes referred to in previous publications were purchased from the same sources [8, 9]. Snake venom dinucleotide nucleotidohydrolase (nucleotide pyrophosphatase, EC 3.6.1.9) and 5′-ribonucleotide phosphohydrolase (5′-nucleotidase, EC 3.1.3.5), as well as *Neurospora crassa* and pig brain NADase, were obtained as lyophilized powders from Sigma, Inc., St. Louis, MO, U.S.A. (-)Cotinine was prepared according to the procedure of Bowman and McKennis [15].

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‡ Abbreviations used in the text are: (nicotine)AD, nicotine adenine dinucleotide; (nicotine)ADP, nicotine adenine dinucleotide phosphate; (cotinine)AD, cotinine adenine dinucleotide; (cotinine)ADP, cotinine adenine dinucleotide phosphate; nicotine(RP), nicotine mononucleotide; and (cotinine)RP, cotinine mononucleotide.

[^3H]nicotine and [^3H]cotinine. [^{3}H]nicotine (2.4 Ci/m-mole) was prepared at New England Nuclear Corp., Boston, MA, U.S.A., by random catalytic tritium exchange on the pyridine ring. It was converted to [^{3}H]cotinine by an enzymic procedure [16]. Minced rabbit liver was homogenized in 0.15 M KCl (1 g tissue/2 ml) and centrifuged at 10,000 *g* for 30 min. The supernatant fraction was used as the liver extract. Ten mCi [^{3}H]nicotine and 0.1 ml of 0.5 M phosphate buffer (pH 7.4) were added to 0.5 ml of the liver extract and diluted to a final volume of 1.0 ml with H_2O. The mixture was incubated at 37° with 2.5 mg NADPH for 1 hr. An additional 2.5 mg NADPH was added and the incubation continued for 1 hr. The reaction was stopped by heating in a boiling water bath for 2 min and the denatured protein was removed by centrifugation. The supernatant fraction was extracted with 3 ml CH_2Cl_2. The [^{3}H]cotinine in the organic layer was purified further by thin-layer chromatography (t.l.c.). In the two successive chromatograms, benzene–acetone–ethanol–NH_4OH (10:8:1:0.4 by vol.) and ethyl acetate–methanol–acetic acid (17:2:1 by vol.) were used as the developing solvents. The R_f values for cotinine were 0.49 and 0.55 and those for nicotine were 0.66 and 0.23, respectively, in these solvents.

Radioimmunoassay. Nicotine and cotinine were determined by the specific radioimmunoassays described by Langone *et al.* [11], except that in the cotinine assay, [^{3}H]cotinine was used as the labeled hapten instead of the [^{125}I]cotinine derivative. In this modified procedure, 0.1 ml [^{3}H]cotinine (approximately 12,000 cpm) was incubated with 0.1 ml of the appropriate dilution of rabbit anti-cotinine sera and 0.1 ml of the sample to be analyzed. After 1 hr at 37°, goat anti-rabbit γ-globulin was added and the immune precipitation was allowed to proceed at 2–4° overnight. The precipitate was collected by centrifugation, dissolved in 0.2 ml 0.1 M NaOH, transferred to scintillation fluid and counted in a liquid scintillation counter.

The specificities of the anti-nicotine and anti-cotinine sera are strict; thus these compounds can be quantitated in the presence of each other and in the presence of other metabolites including cotinine *N*-oxide, desmethyl-cotinine, γ-(3-pyridyl)-γ-oxo-*N*-methylbutyramide, γ-(3-pyridyl)-γ-oxobutyric acid, nicotine *N'*-oxide, and nornicotine [11]. There is essentially no cross-reaction with the nucleoside or nucleotide derivatives of cotinine and nicotine with either anti-serum.

(Cotinine)AD and (cotinine)ADP. Cotinine analogues of NAD and NADP were prepared by procedures similar to those used to obtain (nicotine)AD [8] and (nicotine)ADP [9]. For the preparation of (cotinine)AD, NAD (20 mM), [^{3}H]cotinine (100 mM, 1.0 μCi/m-mole), and sodium phosphate buffer, pH 7.4 (50 mM), were combined in a volume of 50 ml. The pH of the final solution was adjusted to 7.5 with 0.1 N NaOH and the reaction started by the addition of 9 units of pig brain NADase. After 16 hr in a shaking water bath at 37°, the reaction was stopped by heating in a boiling water bath for 3 min and the precipitated protein removed by centrifugation. Cold acetone (200 ml) was added slowly with stirring to the supernatant solution. The resulting precipitate was collected, dried *in vacuo*, then resuspended in H_2O and desalted on a Sephadex G-10 column (2 × 30 cm). Since (cotinine)AD could not be separated from NAD on the Dowex-1 ion exchange resin, it was necessary to treat the crude products with *N. crassa* NADase to hydrolyze unreacted NAD [17, 18]. The lyophilized crude product from the Sephadex G-10 column was dissolved in 30 ml of 50 mM sodium phosphate buffer, pH 7.4, and incubated 16 hr in a 37° water bath with 2.5 units of *N. crassa* NADase. After it was desalted on a Sephadex G-10 column, the product was loaded onto a column (1.5 × 12 cm), packed with Dowex-1 ion exchange resin in the formate form. (Cotinine)AD was eluted from the column by 0.2 M of formate buffer, pH 3.6. About 120 mg (cotinine)AD was obtained with less than 0.1% NAD contamination.

For the preparation of (cotinine)ADP, the incubation conditions were similar to those used for the preparation of (cotinine)AD, except that NADP was substituted for NAD. During the incubation, the amount of NADP which remained in the mixture was determined by the glucose 6-phosphate dehydrogenase assay. When less than 2 per cent of the original NADP could be detected, the incubation mixture was boiled and centrifuged. After precipitation by cold acetone and desalting on a Sephadex G-10 column, the crude product was loaded onto a Dowex-1 column and eluted with a buffer gradient (350 ml H_2O to 350 ml of 0.75 M formate, pH 3.6). Fractions containing (cotinine)ADP were combined, lyophilized and desalted. Dowex-1 column chromatography should be repeated if the contamination of NADP is too high. About 170 mg (cotinine)ADP was prepared with less than 1% NADP contamination.

The separation of nicotine and cotinine analogues of NAD and NADP by an anion exchange column is shown in Fig. 2.

Nicotine mononucleotide. (Nicotine)AD (150 mg) was dissolved in 5 ml buffer (0.05 M Tris–acetate, 0.005 M $MgCl_2$, pH 7.4). Snake venom nucleotide pyrophosphatase (25 units) was added and incubated for 18 hr at 37°. More than 90 per cent of the (nicotine)AD was hydrolyzed under these conditions. The solution was diluted to 100 ml with H_2O, and passed through a Dowex-1 formate column (1.5 × 12 cm). The resin was washed with 50 ml H_2O, and the effluent fluids were combined and lyophilized. The residue was dissolved in 2 ml H_2O and further purified on a Sephadex G-10 column. A solution of 0.05 M NH_4HCO_3 was used as eluant. Fractions which contained (nicotine)RP (measured by absorbance at 260 nm or by [^{3}H]label on nicotine) were pooled and lyophilized. About 70 mg of a hygroscopic solid was collected.

Cotinine mononucleotide. This compound was prepared from (cotinine)AD by a procedure similar to that used to prepare (nicotine)RP from (nicotine)AD. However, when the solution was diluted and passed through the Dowex-1 formate column, the (cotinine)-RP was quantitatively bound by resin. Therefore, a gradient of 100 ml H_2O to 100 ml of 0.2 M formate buffer, pH 3.6, was used as eluant. The (cotinine)RP emerged from the column at a buffer concentration of about 0.08 M. Fractions containing (cotinine)RP (measured by absorbance at 260 nm or by radioac-

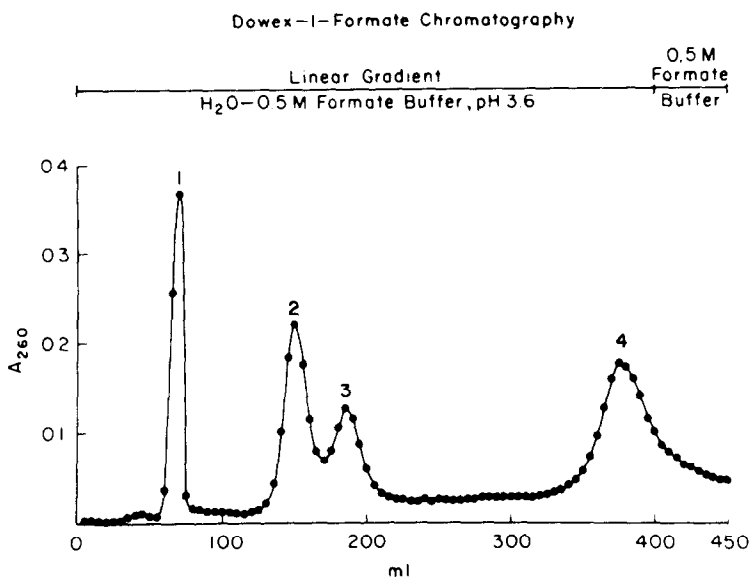

Fig. 2. Separation of the nicotinamide adenine dinucleotide analogues of nicotine and cotinine by ion-exchange chromatography on Dowex-1-formate. A solution (10 ml) containing a mixture of about 0.2 mg of each analogue was applied to the resin column (1.5 × 12 cm). The resin was washed with 30 ml H_2O and then eluted with formate buffer (pH 3.6). Fractions (5 ml) were collected and analyzed by measuring the absorbance at 260 nm. Key: (1) (nicotine)AD, (2) (cotinine)AD, (3) (nicotine)ADP and (4) (cotinine)ADP.

tivity) were pooled, lyophilized and further purified on a Sephadex G-10 column. Final yield was 80 mg of a hygroscopic solid.

Nicotine and cotinine ribosides. About 5 mg of each mononucleotide was dissolved in 0.5 ml buffer (0.05 M glycine, 0.005 M $MgCl_2$, pH 8.5) and 15 units of snake venom 5'-nucleotidase added. After 18 hr at 37°, the solution was boiled for 2 min and centrifuged to remove the precipitate (mostly magnesium phosphate). The clear supernatant solution was passed through a Sephadex G-10 column, and fractions containing the ribosides were collected and lyophilized. Products prepared by this procedure showed only one u.v. absorbing or radioactive spot on t.l.c.

Stability of mononucleotides in rabbit liver extract. The mononucleotides of nicotine, cotinine or nicotinamide were dissolved in a solution containing 0.25 ml of liver extract and 0.25 ml of 0.1 M sodium phosphate buffer, pH 7.4. The final concentration of each mononucleotide was 2 mM. After incubation at 37° for 18 hr, the samples were heated for 1 min in a boiling water bath, diluted with 0.5 ml H_2O and centrifuged to remove denatured protein. Each supernatant fluid was analyzed to determine the extent of hydrolysis. (Nicotine)RP and (cotinine)RP were assayed by measuring radioactivity after t.l.c. NMN was assayed by the cyanide addition reaction [19]. Samples incubated in the absence of liver extracts were used as controls.

RESULTS

Nicotine and cotinine mononucleotide. The products formed after incubation of the nicotine and cotinine analogues of NAD and NADP with snake venom nucleotide pyrophosphatase were separated by t.l.c. (Fig. 3). The cotinine and nicotine analogues of NAD each yielded two products, one of which was identified as AMP by R_f values and spectra. The second product was identical in the hydrolysates of the NAD and NADP analogues of nicotine or of cotinine. Acid hydrolysis of these products yielded nicotine or cotinine. Adenine was absent. This evidence considered together with the known specificity of the enzyme indicates that these products are mononucleotides. Their ability to bind to the anion exchange resin is also consistent with the behavior expected of negatively charged mononucleotides (Table 1). The positive charge on the pyrrolidine ring of nicotine at neutral pH can account for characteristics exhibited by the (nicotine)RP, i.e. its high affinity for the cation exchange resin and somewhat lower affinity for the anion exchange resin.

The corresponding mononucleotide is the major product formed if (nicotine)AD or (cotinine)AD is incubated with a rabbit liver microsomal fraction. In the digests of (nicotine)AD, at least 62 per cent of the nicotine moiety is recovered as (nicotine)RP and 23 per cent as free nicotine (Table 2). Since the (nicotine)RP was recovered by ion exchange chromatography, the actual yield of this product might actually have been greater. With (cotinine)AD, 76 per cent was recovered as (cotinine)RP and 17 per cent as free cotinine. (Cotinine)AD (Fig. 4) and (nicotine)AD were completely hydrolyzed under the experimental conditions used for the enzymic reaction. However, unlike the corresponding cotinine analogues (Fig. 4), (nicotine)AD and (nicotine)RP do not separate satisfactorily on the Dowex-1 resin. Therefore (nicotine)AD digests were analyzed after t.l.c. to determine the extent to which (nicotine)AD had hydrolyzed.

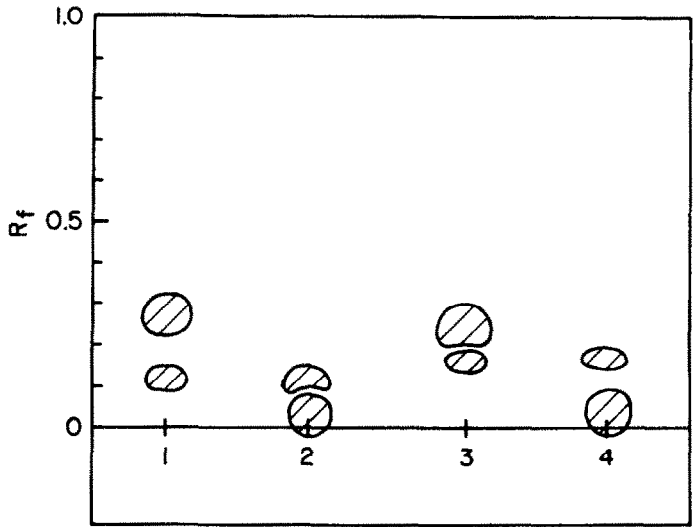

Fig. 3. Thin-layer chromatography of the nucleotide pyrophosphatase hydrolysates of the cotinine and nicotine analogues. Samples from the enzymic hydrolysis of (1) (cotinine)AD, (2) (cotinine)ADP, (3) (nicotine)AD and (4) (nicotine)ADP were applied to a t.l.c. plate and developed in the solvent, 2-propanol–NH_4OH–H_2O (6:3:1 by vol.). After drying in air, the t.l.c. plate was examined under u.v. light. The spot which migrated with an $R_f = 0.27$ was identical to AMP.

The stability of (nicotine)RP and (cotinine)RP in rabbit liver extracts was also studied. After 18 hr of incubation with the liver extract, about 80 per cent of the (cotinine)RP and 33 per cent of the (nicotine)RP were recovered unchanged. The mononucleotides were stable when incubated with buffer under the same conditions. In contrast, NMN was completely hydrolyzed to free nicotinamide during the incubation with liver extract.

Nicotine and cotinine riboside. When incubated with snake venom 5′-nucleotidase, (nicotine)RP and (cotinine)RP are converted to derivatives which can be distinguished by t.l.c. from the dinucleotide analogues, mononucleotides, and free nicotine or cotinine (Fig. 5). Because of the specificity of 5′-nucleotidase [20], these products are assumed to be nicotine and cotinine ribosides. This assumption is supported by their behavior on the ion exchange resins. Products from 5′-nucleotidase digestion possess only positive charges, and therefore bind only to the cation exchange resin. Alkaline hydrolysis converts these ribosides to free nicotine and cotinine.

DISCUSSION

Several enzymes have been implicated in the metabolic turnover of nicotinamide adenine dinucleotides in mammalian tissues [1, 21]. In rat liver microsomes, NADase is the major enzyme responsible for the destruction of NAD. In fact, unless nicotinamide is

Table 1. Binding of the mononucleotides and ribosides of nicotine and cotinine to ion exchange resins*

	Nicotine mononucleotide		Cotinine mononucleotide	
Type of resin	(cpm)	(% bound)	(cpm)	(% bound)
None (control)	4821	0	4866	0
Dowex 1 (anion)	1430	70	856	82
Dowex 50 (cation)	39	99	2269	53
	Nicotine riboside		Cotinine riboside	
	(cpm	(% bound	(cpm)	(% bound)
None (control)	6633	0	6907	0
Dowex 1 (anion)	6200	7	6594	5
Dowex 50 (cation)	38	99	61	99

*The resins, Dowex 1 (formate) and Dowex 50 (H^+), were washed with water and air dried by suction. Each tritium-labeled compound (2–3 mM) in 0.4 ml H_2O (pH 7–8) was mixed with 0.1 g of either resin for 1 min at 25°. After the resin had settled (5 min), 0.2 ml of the clear supernatant solution was removed and counted. The difference in radioactivity between the control and resin-treated sample was used to determine the amount of nucleotide or nucleoside which was bound to each resin. Although 70 per cent of the (nicotine)RP binds to the anion exchange resin at a concentration of 2 mM, lesser quantities bind when more concentrated solutions are chromatographed.

Table 2. Products recovered (per cent of analogue) after incubation of (nicotine)AD or (cotinine)AD with rabbit liver microsomes*

Analogue	Mononucleotide	Nicotine	Cotinine
(Nicotine)AD	62	23	
(Cotinine)AD	76		17

* The reaction mixtures contained either (nicotine)AD or (cotinine)AD (2.5 mM) and liver microsomes (10 mg protein/ml) in buffer (0.05 M sodium phosphate). After 16 hr incubation at 37°, the tubes were placed in a boiling water bath for 2 min and the microsomes removed by centrifugation. Aliquots of the supernatant fraction were used to estimate free nicotine or cotinine by the radioimmunoassay procedure. The remainder of the digests was diluted and added to a Dowex 1 formate column (1.5 × 10 cm) and eluted with a formate buffer gradient (0 to 0.25 M, pH 3.6).

present to inhibit the NADase activity during assay, the cleavage of NAD by nucleotide pyrophosphatase is difficult to detect [21]. (Nicotine)ADP, as well as other nicotine and cotinine analogues, is hydrolyzed very slowly by liver microsomal NADase [8]. The same results were also observed when these nucleotide analogues were incubated with purified pig brain NADase. The specificity requirement of NADase for the nicotinamide moiety in the naturally occurring coenzymes may account for their apparent stability. On the other hand, the relatively broad specificity of nucleotide pyrophosphatase [22] may enable it to play a significant role in the destruction of these dinucleotide analogues. Thus, during incubation of either (nicotine)AD or (cotinine)AD with liver microsomes, about 80 per cent of the nicotine and cotinine moieties was recovered as the corresponding mononucleotides.

The mononucleotides of cotinine and nicotine are relatively stable in the presence of liver extract. This stability may be due to the specificity of enzymes involved in the NMN hydrolysis such as the liver 5′-nucleotidase and other phosphatases.

(Nicotine)RP and (cotinine)RP can be hydrolyzed to their corresponding ribosides by the enzymic action of snake venom 5′-nucleotidase. Dephosphorylation of these compounds could not be detected when liver extracts were used as a source of the enzyme. Recently, the *N*-riboside of 2-hydroxynicotinic acid has been identified as the major urinary metabolite in dogs and rats which have received 2-hydroxynicotinic acid [23]. It is possible that abnormal mononucleotides are dephosphorylated in other tissues, or that the liver nucleotidase is more specific for 2-hydroxy-nicotinic acid mononucleotide than for the (nicotine)RP and (cotinine)RP. Species variations in such enzymes may also exist.

Because of their charge, it is generally believed that the ability of nucleotides to penetrate through cell membranes is low [24, 25]. Our results suggest that (nicotine)RP and (cotinine)RP may accumulate inside the cell in some rabbit tissues. This assumption is supported by our studies *in vivo* which indicate that

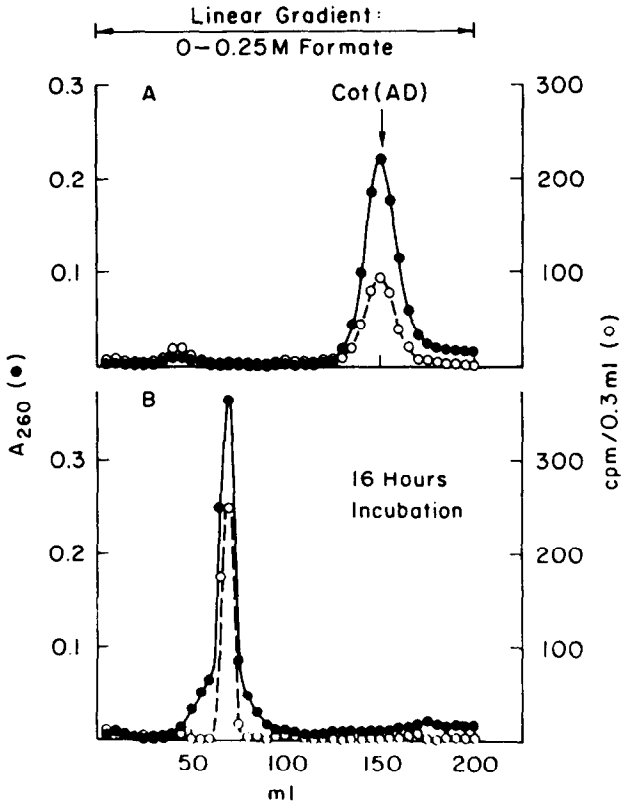

Fig. 4. Hydrolysis of (cotinine)AD by a rabbit liver microsomal fraction. (A) Before incubation; (B) after 16 hr incubation. The experimental conditions are described in Table 2. The (cotinine)AD digest was diluted, applied to a Dowex-1-formate column (1.5 × 10 cm) and eluted with formate buffer.

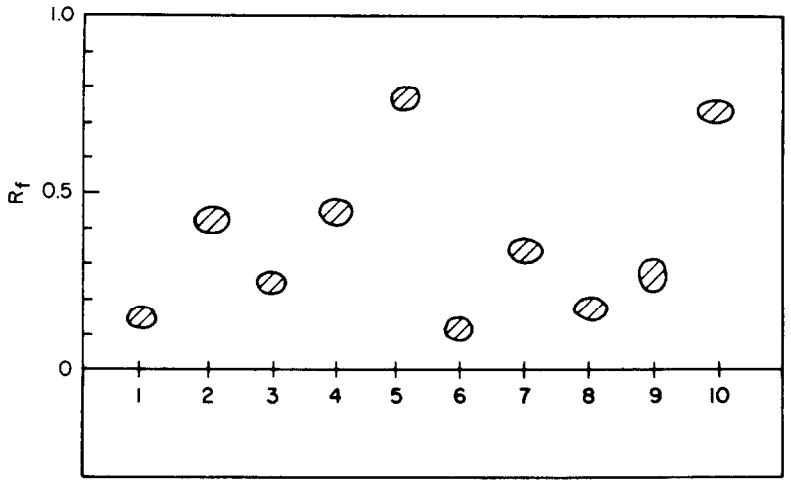

Fig. 5. Thin-layer chromatogram of the nucleotide and nucleoside analogues of nicotine and cotinine developed in 2-propanol–NH_4OH–H_2O (6:3:1 by vol.). Key: (1) (nicotine)AD, (2) (nicotine)ADP, (3) (nicotine)RP, (4) nicotine riboside, (5) nicotine, (6) (cotinine)AD, (7) (cotinine)ADP, (8) (cotinine)RP, (9) cotinine riboside and (10) cotinine.

(cotinine)RP is the major nucleotide analogue in the liver and lung of rabbits injected with either nicotine or cotinine [26]. Even though the analogues may account for a minor proportion of the compounds which result during the metabolism of nicotine, they are potentially among the most important.

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