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Formation *in Vitro* and *in Vivo* of the Isonicotinic Acid Hydrazide Analogue of Nicotinamide Adenine Dinucleotide by Lung Nicotinamide Adenine Dinucleotide Glycohydrolase

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SUMMARY

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Mammalian lungs have relatively high levels of NAD glycohydrolase activity. The enzyme in this organ appears to occur exclusively in membrane fractions with high activities in the 25,000 × *g* and 105,000 × *g* sediments. Isolated rabbit pulmonary alveolar macrophages exhibited no enzyme activity, either as intact cells or as sonicated suspensions. The membrane-bound enzyme from rat lung was shown to have a broad pH optimum (5.9–6.9) and low NADP glycohydrolase activity. The enzyme was "insensitive" to isoniazid (INH), and, among a group of congeners tested, only nicotinamide, a reaction product, was a potent inhibitor. Transglycosidase activity *in vitro*, as measured spectrophotometrically and chromatographically, was observed in the presence of INH. Formation of the INH analogue of NAD *in vitro* was inhibited by nicotinamide. To examine lung transglycosidase activity *in vivo*, [^{14}C]INH was injected intravenously into rats and the lungs were extracted and analyzed for the oxidized nucleotide analogue. Identification of significant levels of isotope covalently linked in the nucleotide support transglycosidase activity *in vivo*. The half-life of the analogue ($t_{1/2}$ ~ 60 min) in the lung was approximately twice that of the total organ ^{14}C decay rate ($t_{1/2}$ ~ 28 min).

INTRODUCTION

The demonstration of different enzymes in animals and microbes that degrade NAD at various bonds in this cofactor is well established. From the studies of Handler and Klein in 1942, a glycohydrolase (EC 3.2.2.5) was shown to exist in mammalian tissues which degraded NAD at the nicotinamide-ribose linkage to yield free nicotinamide and adenosine diphosphate ribose (1). These enzymes are essentially unable to hydrolyze the same bond in the reduced form of NAD and have been subsequently known as "NADases." The reverse reaction, which acts as a transglycosidase, is also well known and has been employed to make different analogues of this coenzyme (2). For example, the biosynthesis of the isonicotinic acid hydrazide analogue of NAD has been achieved *in vitro* using NAD glycohydrolase (3, 4). An adenosine diphosphate ribose–enzyme intermediate has been proposed to account for the reversibility and isotopic exchange observed in the presence of selected analogues of nicotinamide (3, 4).

The lung has been previously shown to be a rich source of this enzyme (5), and

during infection with *Mycobacterium tuberculosis* the level of this nucleosidase is elevated in this organ with the concomitant reduction of NAD levels (6). These reported observations have led me to examine properties of the lung membrane-bound glycohydrolase. Since the INH[1] analogue of NAD cannot function as an electron carrier in dehydrogenase reactions *in vitro* (7), an investigation was made to determine whether transglycosidase-catalyzed isotopic exchange of INH for nicotinamide occurs within the intact animal.

MATERIALS AND METHODS

All chemicals were of commercial origin and, when possible, reagent grade. β-NAD, α-NAD, INH, and congeners of nicotinamide were purchased from Sigma Chemical Company. [*carbonyl*-^{14}C]Isonicotinic acid hydrazide (specific activity, 11.1 mCi/mmole) and [*carbonyl*-^{14}C]nicotinamide (specific activity, 60.0 mCi/mmole) were purchased from Amersham/Searle.

Adult (200–250-g) male rats of the Sprague-Dawley strain were obtained from Charles River Laboratories and fed ad libitum. The animals were killed by a sharp blow on the head, and the lungs were quickly removed, rinsed in chilled 0.25 M sucrose, blotted, minced, and compressed through an Arbor tissue press (model 142, Harvard Apparatus Company). The tissue was then homogenized in 4 volumes of ice-cold 0.25 M sucrose containing 1 mM $MgSO_4$, using a Potter-Elvehjem apparatus immersed in cracked ice and equipped with a Teflon pestle. The homogenate was strained through six layers of cotton gauze. Differential centrifugation of the remaining material was carried out in a manner similar to that previously reported (8).

Marker enzymes were assayed in the manner described in the references: glucose 6-phosphatase (9), succinate–cytochrome *c* reductase (10) and 5′-nucleotidase (11).

[1] The abbreviations used are: INH, isonicotinic acid hydrazide (isoniazid); TCA, trichloracetic acid; ARPPR, the adenosine moiety (adenosine diphosphate ribose) of NAD.

Rabbit alveolar macrophages were collected from the lung according to the method of Myrvick *et al.* (12). Only preparations essentially free of erythrocytes were used.

Measurement of NAD or NADP glycohydrolase activity. The cyanide adduct method used for determining NAD glycohydrolase activity was similar to that reported by Kaplan (13). Except where otherwise stated, appropriate tissue fractions were incubated with 1.5 mM NAD in 0.1 M phosphate buffer at pH 6.2 (final volume, 2.5 ml) for 20 min at 37°. Reactions were terminated by adding 0.5 ml of 24% (v/v) perchloric acid and centrifuging the suspension. One milliliter of the supernatant fraction was withdrawn and added to 3 ml of 1 M KCN; this mixture was centrifuged and the absorbance of the supernatant was read at 325 nm.

Measurement of enzyme-catalyzed NAD-INH exchange in vitro and in vivo. The exchange reaction was measured spectrophotometrically in a manner similar to the method reported by Zatman *et al.* (4). The assay *in vitro* contained NAD, 1.5 mM; INH, 2.0 mM; 1 mg of fraction II (25,000 × *g* pellet), measured as protein; and 0.1 M phosphate buffer, pH 6.2, in a total volume of 2.5 ml. Chromatographic evidence for analogue formation in the incubated media was obtained by spotting an aliquot (10–50 μl) of the reaction mixture containing 2 mM [*carbonyl*-^{14}C]INH (specific activity, 0.5 mCi/mmole) on Whatman No. 4 paper and developing the chromatogram with an ascending solvent system containing ethanol–0.1 N acetic acid (1:1 v/v) in a lined chamber. The solvent was allowed to travel 20 cm, and the chromatogram was air-dried in the hood for about 1 hr and finally cut into rectangular strips for counting. The strips were added to vials containing 1 ml of water and mixed well. Then 10 ml of Aquasol (New England Nuclear) scintillation mixture were added, and the samples were counted in a Beckman scintillation counter.

The presence of the INH analogue of NAD in the lung *in vivo* was determined by injecting approximately 4 μCi of [^{14}C]INH (specific activity, 11.1 mCi/

mmole) dissolved in 0.9% NaCl solution (0.2 ml) into the tail veins of rats of nearly identical body weights. At appropriate intervals following injection, the rats were killed and their lungs were quickly excised, blotted, weighed, finely minced in an ice-chilled beaker, and washed with cold 0.9% NaCl to remove as much residual blood as possible. The mince was further dispersed by sonication (four 10-sec bursts) in 4 volumes of cold 10% (w/v) TCA, and then homogenized (eight passes) with a Potter-Elvehjem apparatus. The mixture was centrifuged, and the supernatant fraction was removed to another vessel. Two milliliters of 5% TCA were added to the pellet, the contents were mixed well to obtain a uniform suspension, and the above procedure was repeated. The pooled TCA extracts were mixed well with 4 volumes of acetone and stored for 48 hr at −20°. Free INH remained completely soluble under these conditions. The acetone-TCA mixture was centrifuged, and all but a small volume of the resulting supernatant fraction was removed by aspiration. The precipitate was washed further with three 2-ml volumes of acetone, and the small volume of solvent left above the precipitate after the last washing was evaporated with a stream of N_2. The precipitate was then dissolved by four 1-ml sequential additions of water to the scintillation vials, and lyophilized. The residue obtained by lyophilization was dissolved in Aquasol and counted by liquid scintillation. No reduced dinucleotide (NADH or NADPH) could be detected by ultraviolet absorbance in aqueous solutions of the acetone-TCA pellet. The total amount of extracted oxidized nucleotide (NAD plus NADP) varied less than 4% (nanomoles per gram ± standard error) among different animals, and injection of [^{14}C]INH effected no significant change in oxidized nucleotide levels.

For the determination of total lung ^{14}C activity, the washed lungs, prepared as described above, were minced and then homogenized in 2 volumes of ice-cold 0.9% NaCl. The samples were frozen until analysis. A 0.5-ml aliquot of each sample was combusted (Harvey Instrument Corporation), and $^{14}CO_2$ was trapped in a 10-ml solution of toluene–ethylene glycol monomethyl ether–ethanolamine (6:7:7, v/v/v). The trapping column was rinsed with 10 ml of a solution containing toluene–ethylene glycol monomethyl ether–scintillator (Research Products International) (12:5:3, v/v/v). The trapping solution and scintillator mixture were combined after combustion, mixed well, and counted. Measurement of isotopic activity in blood samples taken at the time of death indicated that less than 5% of the total organ activity could be accounted for by [^{14}C]INH present in the calculated volume of residual blood in excised lungs.

RESULTS

Subcellular localization. Differential centrifugation of the homogenized lung in the $MgSO_4$-sucrose solution revealed that NAD glycohydrolase was localized nearly exclusively in the sedimentable material from the lung (Table 1). High specific activities were observed in the 25,000 × *g* (fraction II) and 105,000 × *g* (fraction III) pellets. Fraction IV, or the cytosol fraction, had virtually no NAD glycohydrolase activity, and when this fraction was recombined with fraction II or fraction III, no inhibition of glycohydrolase activity was observed. Despite the high relative activity of the mitochondrial marker succinate–cytochrome *c* reductase, electron photomicrographs of fraction II revealed numerous elongated, nonvesiculated membranes and vesicles of rough endoplasmic reticulum. In further subfractionation studies[2] a preparation enriched in rat lung mitochondria was found to have a significantly lower specific activity of NAD glycohydrolase than the fraction II pellet. The enzyme specific activity appeared to parallel that of glucose 6-phosphatase (Table 1), suggesting that this lung enzyme may be associated with the endoplasmic reticulum.

Sedimentable fractions obtained from rabbit lung homogenates were also shown to be rich in glycohydrolase activity (Table 2). Alveolar macrophages, obtained in high purity by lavage of rabbit lungs, were found to have no detectable levels of the

[2] Unpublished observations.

TABLE 1

Subcellular distribution of rat NAD glycohydrolase and marker enzymes

Isolation and incubation conditions are explained in detail in MATERIALS AND METHODS. All figures are averages of values from five analyses done in duplicate.

Fraction	NAD glycohydrolase	Glucose 6-phosphatase	5'-Nucleotidase	Succinate–cytochrome *c* reductase
	μmoles/hr/mg protein	*nmoles/min/mg protein*	*nmoles/min/mg·protein*	*nmoles/min/mg protein*
Homogenate	1.3			
I.1,000 × *g* sediment	1.9	1.1	2.6	5.4
II.25,000 × *g* sediment	7.0	2.8	9.8	43.8
III.105,000 × *g* sediment	5.5	2.0	14.4	4.0
IV.105,000 × *g* supernatant (cytosol)	0.1	0.1	0.8	0
Fraction II plus cytosol (10 mg)	7.1			
Fraction III plus cytosol (10 mg)	5.6			

enzyme, either as intact cells or as sonicated cellular suspensions. When the sonicated alveolar macrophages from the pellet were combined with the rabbit 25,000 × *g* pellet, no inhibition of enzyme activity in this fraction resulted (Table 2).

Some properties of the membrane-bound enzyme. The pH-activity curve for the membrane-bound rat lung glycohydrolase revealed a broad optimum between pH 5.9 and 6.9. When NADP, rather than NAD, was used as substrate within this pH range, only about 10% of the activity obtained with NAD was observed. As expected, no glycohydrolase activity was present when NADH or NADPH was used as substrate. When the α-anomer of NAD was substituted for β-NAD, no metabolized NAD could be detected by the cyanide adduct method. As shown in Table 3, some congeners of nicotinamide were examined for their capacity to inhibit rat lung glycohydrolase activity. Nicotinic acid, isonicotinic acid and isonicotinamide were very weak inhibitors of the reaction when compared to the parent nicotinamide molecule. The low degree of inhibition given by isoniazid establishes the lung enzyme as isoniazid-"insensitive."

Formation of INH analogue of NAD by lung tissue. The incubation of NAD glycohydrolase and its substrate NAD in the presence of isoniazid was shown previously by other workers to yield a product having an absorbance maximum at 390 nm in alkaline media (3). Much chemical evidence has been published to show that the ARPPR-IHN analogue of NAD is responsible for the formation of this colored product.

TABLE 2

NAD glycohydrolase activity of rabbit lung homogenate, 25,000 × g pellet (fraction II), and alveolar macrophages

Isolation and assay procedures are explained in detail in MATERIALS AND METHODS. All figures are averages of values from three analyses done in duplicate.

Conditions	NAD metabolized
	μmoles/hr/mg protein
Alveolar macrophages	0
Sonicated alveolar macrophages	0
25,000 × *g* pellet from rabbit whole lung	2.5
Sonicated 25,000 × *g* pellet from rabbit whole lung	2.4
Sonicated alveolar macrophages + sonicated 25,000 × *g* pellet	2.4
Lung homogenate	0.6
Lung homogenate (sonicated)	0.6

Incubation of the rat lung 25,000 × *g* pellet with fixed amounts of INH and various concentrations of NAD resulted in the formation of the 390 nm-absorbing product, confirming that the lung membrane-bound enzyme can act as a transglycosidase and exchange INH for nicotinamide in the presence of the nucleotide. The same reaction, containing NAD and INH but no enzyme, yielded no yellow product. When NAD was replaced with NADP in this reaction, only negligible amounts of

TABLE 3

Effects of congeners of nicotinamide on rat lung membrane-bound (25,000 × g pellet) NAD glycohydrolase

The assay procedure is described under MATERIALS AND METHODS. The concentration of NAD was 1.5 mM. Samples were incubated at 37° for 20 min. All figures are averages of values from three analyses done in duplicate.

Compound	Concentration	NAD metabolized	Inhibition
	mM	*μmoles/hr/mg protein*	%
None		7.0	
Nicotinamide	1	4.2	40
	2	3.0	57
	5	1.2	83
Isonicotinamide	1	5.7	19
	2	5.4	23
	5	4.5	36
Nicotinic acid	5	6.3	10
Isonicotinic acid	5	7.0	0
INH	1	6.5	7.1
	2	4.9	30
	5	3.7	47

the INH analogue were formed. To show further that the formation of the ARPPR-INH analogue was glycohydrolase-dependent, various concentrations of nicotinamide were added to a reaction mixture containing the bound enzyme, NAD, and INH. As depicted in Fig. 1, nicotinamide effectively inhibited formation of the ARPPR-INH analogue and by plotting nicotinamide concentration against absorbance at 390 nm, a straight line was obtained. This linearity could be taken as evidence for competition between INH and nicotinamide for the adenosyl ribose pyrophosphate ribose–enzyme (ARPPR) complex.

Further evidence for the formation *in vitro* of the analogue made with INH came from isotope exchange studies using [*carbonyl*-^{14}C]INH. In this series of experiments isotopic INH was added to a reaction mixture containing the rat lung 25,000 × *g* pellet and buffer (pH 6.2). After incubation, an aliquot of the reaction was withdrawn and subjected to paper chromatography. Zonal sections of the chromatogram revealed a single isotopic peak with an R_F of approximately 0.8 (Fig. 2). When the same procedure was repeated following the inclusion of NAD in the reaction mixture, a second isotopic, trailing peak was obtained. This second peak consisted of about 10% of the total isotopic activity on the chromatogram. The activity in this second peak accounted for the decrease in area that was observed in the leading major peak when NAD had been omitted. The isotopic material formed in the presence of NAD and [^{14}C]INH had the same R_F as the unknown detected on paper chromatograms under ultraviolet light using the same incubation conditions, without isotope. Elution of the unknown material from chromatograms gave a material that had an absorbance maximum near 390 nm in alkali (0.1 N NaOH). Inclusion of excess nicotinamide (5 mM) in the reaction mixture prevented the formation of the trailing minor peak shown in Fig. 2.

The major aim of this study was to observe whether or not ARPPR-INH formation by lung occurred *in vivo* and, if so, how its half-life would compare with that of isoniazid. To investigate analogue formation *in vivo*, [^{14}C]INH was injected in-

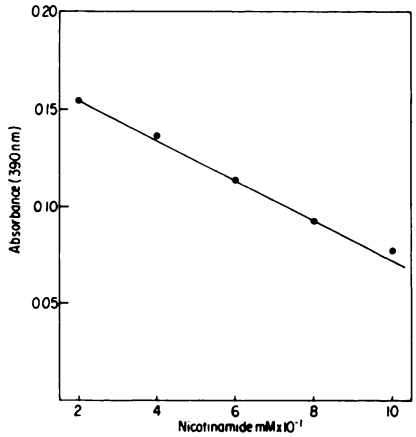

FIG. 1. *Relationship between nicotinamide concentration and inhibition of ARPPR-INH formation by rat lung membrane-bound NAD glycohydrolase*

Fraction II (1 mg of protein) of the rat lung homogenate was incubated with NAD, 1.5 mM; INH, 20 mM; phosphate buffer, 0.1 M, pH 6.2; and various amounts of nicotinamide as indicated. Incubations were carried out for 20 min at 37°, and analogue formation was determined by the method of Zatman *et al.* (3).

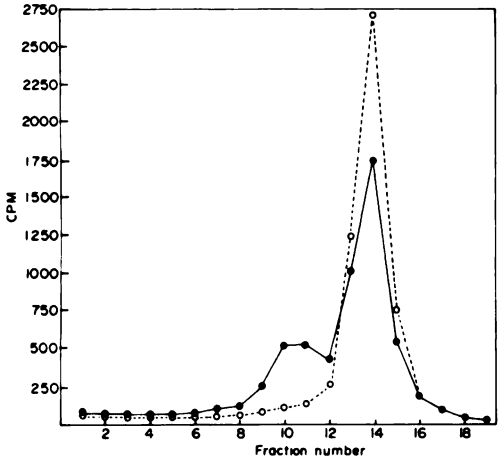

FIG. 2. *Formation of INH analogue of NAD, ARPPR-INH, in vitro by rat lung NAD glycohydrolase*

●——●, an aliquot (10 μl) taken from a reaction containing phosphate buffer, 0.1 M; NAD, 1.5 mM; [*carbonyl*-^{14}C]INH, 2 mM; and 1 mg of protein of fraction II (25,000 × *g* pellet). The mixture was incubated for 20 min. The aliquot was withdrawn, spotted on Whatman No. 4 paper, and developed in ethanol–0.1 N acetic acid (1:1, v/v). Fraction 19 represents the solvent front at a migration distance of 20 cm. O- - -O, results obtained when NAD was omitted from the reaction.

travenously into rats, and the animals were killed at intervals in order to determine total lung ^{14}C and ARPPR-[^{14}C]INH activities. As shown in Fig. 3, a significant portion of the ^{14}C activity was present in the nucleotide fraction isolated from the lung. The radioactivity of this extracted material co-chromatographed with the minor trailing peak discussed above and shown in Fig. 2. The analogue formed *in vivo*, as depicted by the lower line in Fig. 3, had a half-life of about 60 min; total organ radioactivity obtained by whole tissue oxidation, as shown in the upper plot of this figure, decayed about twice as fast as the analogue ($t_{1/2}$ = 25–30 min). When the same experiment was repeated using isotopic nicotinamide injected intravenously, subsequent extraction of NAD from the whole lung revealed rapid uptake with a nearly constant level of isotopic specific activity throughout the time course period.

DISCUSSION

Since the discovery of INH as a potent antitubercular drug, numerous investigations have been made to uncover the primary mechanisms of action of this compound (14). A metabolic characteristic of many *M. tuberculosis*-infected organs, such as the lung, is a distinct elevation of NAD glycohydrolase activity (6). This elevated activity generally returns to normal following INH chemotherapy (15). A previous study indicated that suppression of the elevated NAD glycohydrolase activity by INH is due to its antimycobacterial activities and not to some specific action on host metabolism (15). In those cases when the enzyme is INH-"insensitive" (3), i.e., not capable of being inhibited by INH, exchange can occur between the drug and the nicotinamide moiety of NAD. Although the "sensitive" enzyme is strongly inhibited in INH, analogue formation still occurs, but at a much slower rate (4). Since

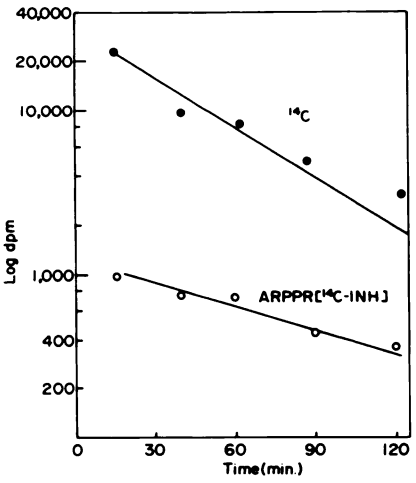

FIG. 3. *Formation of INH analogue of NAD in rats in vivo*

[*carbonyl*-^{14}C]INH was injected intravenously into rats, and the isotopic incorporation into total lung (●) and exchange with NAD (O) are shown at selected times following injection. The ordinate represents the ^{14}C per gram of lung and ARPPR-[^{14}C]INH extracted per gram of lung, respectively. Detailed procedures are given in MATERIALS AND METHODS. The total amount of extracted oxidized nucleotide varied less than 4% (nanomoles per gram ± standard error) among different animals.

the INH analogue of NAD cannot participate readily in reactions where NAD is involved in dehydrogenase reactions, the formation of this INH analogue of NAD has been postulated as a tuberculostatic mechanism of action for INH (16).

According to previous studies, about two-thirds of the NAD in the rat lung is present in the oxidized form. There is also 6 times more NAD than NADP, and most of the NADP is present in the reduced state (17). These conditions should favor formation *in vivo* of the ARPPR-INH analogue in the presence of the glycohydrolase and INH.

The approximately 2-fold longer half-life of the ARPPR-[^{14}C]INH analogue when compared to the total lung ^{14}C activity contributed by [^{14}C]INH injection could be accounted for by at least two factors. (a) Once formed, the INH-NAD analogue is not likely to diffuse or exit from the lung as an intact molecule to the same extent as isoniazid or its metabolites, e.g., acetylisoniazid or isonicotinic acid (18). (b) The cleavage of the ribose-isoniazid bond of the analogue is thought to occur much more slowly than that of the original cofactor ribose-nicotinamide bond (4). The very low glycohydrolase activity found in blood and the early onset of decay of the INH-NAD analogue *in vivo* make it unlikely that the analogue is first formed in some other tissue and then transported to the lung. As judged from the experiments of others (19), it is unlikely that NAD or the analogue nucleotide would be able to be transported into the cell from the serum or extracellular spaces without first being degraded by membrane pyrophosphatases or glycohydrolases (20), for example.

When [7-^{14}C]nicotinamide was injected into rats in the same manner as the isotopic INH, rapid uptake and negligible turnover were noted in the 6 hr during which isotopic activity was measured in the [^{14}C]NAD extracted from whole lung. A similar result was noted in a previous study when the incorporation of this isotope into brain NAD was measured following cisternal injection of [^{14}C]nicotinamide (19). Since the reduced form of NAD is not degraded by the glycohydrolase, it is quite possible that rapid reutilization of NAD in oxidation-reduction reactions maintains a slow rather than a rapid turnover of the nicotinamide moiety of this cofactor. However, other possible factors, such as the rapid reutilization of free nicotinamide, should also be considered.

Judging by the ratio of ARPPR-[^{14}C]INH to total ^{14}C in the lung during the early periods of the time course experiments following [^{14}C]INH injection, it appears likely that an appreciable amount of the total isoniazid in the lung can exist as ARPPR-INH following the dose regimen of INH used (8–12 mg/kg daily) (21) for *M. tuberculosis* infection and prophylaxis. However, further studies are required to determine whether the presence of the analogue in infected tissue is causally related to the tuberculostatic action of INH.

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