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Drug–Biomolecule Interactions: Drug Toxicity and Vitamin Coenzyme Depletion

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Abstract ☐ Thirteen pyridine compounds, phenylbutazone, and three salicylates were studied for their effects upon the turnover of 7-^{14}C-nicotinamide dinucleotides in the mouse. The compounds were administered at equitoxic doses (LD_{25}) to 7-^{14}C-nicotinic acid- (niacin) pretreated mice, and the induced excretion of urinary-^{14}C was analyzed in terms of total ^{14}C and percentage of total ^{14}C as known metabolites of nicotinic acid. Of the 17 compounds, 12 afforded significant alterations in the total ^{14}C excreted and five of these caused alterations in the disposition of the 7-^{14}C-nicotinamide endogenously liberated from the 7-^{14}C-nicotinamide adenine dinucleotide pool. Comparative depletions of ^{14}C from brain, lungs, liver, and kidneys were studied with 10 of the pyridine compounds. Several tissues were found to be the sources of the urinary-^{14}C, with the lungs being the most accessible source. Some compounds had effects at doses less than the LD_{25}'s, as shown by increased hexobarbital sleeping time in acute experiments with rats. These pyridine compounds were initially considered to act at the level of the nicotinamide dinucleotides in the normal biosynthetic pathway (nicotinic acid site) and/or at the level of glycohydrolase (nicotinamide site). In view of the inclusion of nicotinic acid, nicotinamide, salicylic acid, and phenylbutazone in this correlation between toxicity and 7-^{14}C-nicotinamide mobilization, it is not necessary that the formation of compounds analogous to the nicotinamide dinucleotides plays a significant role in the toxic manifestations of the nicotinamide analogs. The displacement of 7-^{14}C-*nicotinamide* dinucleotides from their corresponding apoenzymes with subsequent metabolism of the dinucleotides could explain the noted increased 7-^{14}C-nicotinamide dinucleotide turnover and depletion which led to the toxic effects.

Keyphrases ☐ Coenzyme depletion—effects of 17 compounds, motor activity, sleeping time, toxicity, therapeutic effects, coenzyme binding site ☐ Toxicity—pyridine compounds, salicylates, phenylbutazone, nicotinic acid site, nicotinamide site, coenzyme binding site ☐ Metabolism—nicotinamide, coenzymes, nicotinamide dinucleotides, apoenzymes ☐ Drug-biomolecule interactions—drug toxicity and vitamin coenzyme depletion, symposium ☐ Interactions—drugs with biomolecules, symposium

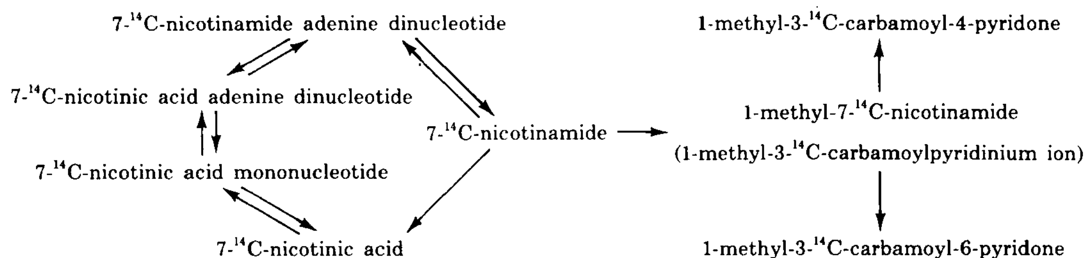
Since initial toxicity studies (1, 2), McDaniel *et al.* (3) described niacin and seemingly anomalous antiniacin or toxic effects of 3-acetylpyridine and several attempts have been made to reconcile such disparities. Thus, antiniacin properties were considered in compounds related to nicotinic acid (niacin) and nicotinamide (niacinamide) (4–6). Since these studies with nicotinamide and some possible antimetabolites, attention has been focused on the nicotinamide dinucleotides[1] (NAD and NADP). The production of analogs and/or alterations in the concentrations of these dinucleotides have been considered as possible explanations of the antiniacin effects of 3-acetylpyridine (5–9) and of 6-aminonicotinamide (10).

It has been reported that nicotinamide administration results in increased levels of liver NAD, with maximal increases at 8–12 hr after the administration of 250 mg/kg ip to the mouse (11) and 1 g/kg ip to the rat (12). The latter study showed a relationship among the increased nicotinamide (23-fold), nicotinic acid (65-fold), and NAD (11-fold) levels in the liver after nicotinamide administration. This induced synthesis is in accord with the normal biosynthetic pathway (Scheme I), wherein the *in vivo* hydrolysis of nicotinamide would furnish the nicotinic acid.

Moreover, compounds other than nicotinic acid and nicotinamide have been reported to increase liver NAD levels. Kaplan *et al.* (11) showed that the administration of 3-methylpyridine (β-picoline) afforded a higher liver NAD level and more rapid rise to maximal effect than did nicotinamide. This increase could possibly be explained as a consequence of the continuous *in vivo* oxidation of 3-methylpyridine to nicotinic acid, with resultant NAD biosynthesis. Alternatively, and perhaps concomitantly, the 3-methylpyridine could cause a general tissue depletion of NAD whose mobilization could lead to the liver levels of NAD described.

Nicotinamide and related substances lend themselves readily to studies relating biological effect and biochemical change, since the normal biotransformations of nicotinamide have been intimately defined in regard to NAD biosynthesis (13) and to nicotinamide

[1] The phrase nicotinamide dinucleotides will include the oxidized [NAD (nicotinamide adenine dinucleotide) and NADP (nicotinamide adenine dinucleotide phosphate)] and corresponding reduced forms (NADH and NADPH) since quantitative assessments of the amounts of the individual substances have not been performed.



Scheme I—Biotransformation of 7-^{14}C-nicotinic acid and some urinary metabolites of 7-^{14}C-nicotinamide dinucleotide-derived 7-^{14}C-nicotinamide in the mouse

metabolism (14–17). This report describes a relationship between the acute toxicities of nicotinamide, some analogous compounds, and some highly protein bound drugs and their abilities to alter the 7-^{14}C-nicotinamide turnover from the labeled nicotinamide dinucleotides in the 7-^{14}C-nicotinic acid-pretreated mouse.

EXPERIMENTAL

Chemicals—The 7-^{14}C-nicotinic acid[2] (11 mCi/mmole) exhibited a single radioactive zone at R_f 0.2 in Solvent H. The 3-(1-hydroxyethyl)pyridine was prepared according to McKennis *et al.* (18). All other chemicals were obtained from commercial sources, and the liquids were purified by distillation prior to use.

Animals—Young adult female Swiss albino and Charles River mice, 20–32 g, and female Sprague–Dawley rats, 140–170 g, were used. The animals were housed at ambient conditions and allowed food and water *ad libitum* unless otherwise indicated.

Chromatography—Paper[3] chromatograms were developed by the descending method at ambient temperatures in Solvent H [upper phase from saturated aqueous 2-methyl-1-propanol–concentrated aqueous ammonia (50:1 v/v)] (compound and R_f): 1-methylnicotinamide, 0.07; nicotinic acid, 0.18; 1-methyl-3-carbamoyl-4-pyridone, 0.34; 1-methyl-3-carbamoyl-6-pyridone, 0.47; and nicotinamide, 0.69.

Acute Toxicity Determinations—The chemicals were administered intraperitoneally in a volume of 0.2 ml. Pyrazinamide, thionicotinamide, and phenylbutazone were administered in dimethyl sulfoxide, and isoniazid and nicotine were administered in pH 4 buffer solution[4]. Nicotinic acid and the salicylates were dissolved in aqueous sodium hydroxide, and all other chemicals were administered in aqueous solution. The LD_{25} values were calculated according to the method of Litchfield and Wilcoxon (19) after a 7-day observation period with either five or six groups of 10–30 mice per treatment.

Treatment with 7-^{14}C-Nicotinic Acid and Other Agents—Groups of five mice received 7-^{14}C-nicotinic acid (3.3 μg, 0.29 μCi) and were maintained under normal conditions for 24 hr. The mice were then divided into control and treatment groups and were manipulated before being transferred to obtain evacuation of the urinary bladder. This manipulation involved squeezing the mouse between the thumb and index finger at areas just anterior to the hindlimbs, with gentle pulling of the mouse clinging to a horizontal metal screen.

The mice were then treated with the test chemicals at a dose equal to the LD_{25} and placed into metabolism cages. Urine was collected from both the control and treated groups again, with appropriate manipulation to obtain bladder evacuation at the end of the 16-hr experiments.

Radioactivity Measurements—The scintillation solution contained 5.0 g of 2,5-diphenyloxazole and 0.3 g of *p*-bis[2-(5-phenyloxazolyl)]benzene in 1 liter of toluene. Samples were counted in scintillation solution containing 15 ml of cocktail and 3 ml of methanol. Urinary aliquots (0.1 ml) from mice pretreated with 7-^{14}C-nicotinic acid were added directly to the scintillation solution for counting.

Paper chromatograms of urinary aliquots were sectioned at 1-cm intervals perpendicular to their long axes, and each section was placed in the scintillation solution for counting. In this manner, the position (R_f) of the radioactivity on the paper could also be obtained.

Determination of Tissue-^{14}C—At the conclusion of the 16-hr experiments, the mice were sacrificed with ether. The carcasses were then maintained at about −10° until analyzed for ^{14}C. The specific tissue samples (brain, kidneys, liver, and lungs) from all animals (four or five) in each group were combined and placed in 3% sodium hydroxide solution (1 g of tissue/5 ml of solution). Dissolution was accomplished by shaking the tissue suspension contained in 20-ml glass vials overnight at ambient temperatures.

To 2 g of the tissue digest were then added a solubilizer[5] (3 ml), to obtain a pH of about 7, and 15 ml of Bray's scintillation solution (for aqueous samples: naphthalene, 60 g; 2,5-diphenyloxazole, 4 g; *p*-bis[2-(5-phenyloxazolyl)]benzene, 0.2 g; methanol, 100 ml; ethylene glycol, 20 ml; and dioxane to 1 liter).

Gross Motor Activity—The activities of the mice were quantitated in a photoactivity cage[6], and cumulative counts were obtained during 30 min after the intraperitoneal administration of each compound to duplicate groups of five mice. Control counts were obtained on different mice that received only the vehicle.

Sleeping Times—Groups of eight–12 animals were used at each dose of hexobarbital (Tables I and II). Sleeping times were recorded as the time in minutes between the loss and return of the righting reflex.

RESULTS

Toxicity—The toxicity studies afforded the LD_{25} values shown in Tables II and III. The ratios of LD_{75}/LD_{25} shown in Table III indicate a very narrow range of slopes (1.14–1.85) for the various dose–response curves, except with thionicotinamide (3.42). Although the observed range of slopes is narrow, the lines cannot be considered parallel except for the group including 3-cyanopyridine, 3-pyridinecarboxaldehyde, pyrazinamide, 3-acetylpyridine, and isoniazid according to the method of Litchfield and Wilcoxon (19).

During the 7-day observation periods, the mice died mainly between 10 and 72 hr after receiving the test chemicals at a dose equal to the LD_{25}. With nicotine and isoniazid, however, the susceptible mice died within a few minutes to less than 3 hr and no further deaths were noted in the ensuing 7 days. The general toxic signs covered the spectrum, from essentially immobile mice with thionicotinamide to opisthotonus with nicotine.

Phenylbutazone, the three amides (nicotinamide, pyrazinamide, and thionicotinamide), and the salicylates produced only sedation; at the higher doses of pyrazinamide and thionicotinamide, paralysis of the posterior extremities was noted. With 3-cyanopyridine and 3-pyridylcarbinol, a slight stimulatory effect followed by a period of brief inactivity was seen within 1 hr after administration. However, only hyperactivity and frequent convulsions were seen with isoniazid and nicotine. After the administration of doses of 3-(1-hydroxyethyl)pyridine and 3-acetylpyridine near the LD_{50}

[2] New England Nuclear Corp.
[3] Whatman No. 1.
[4] Beckman No. 3506.
[5] Bio-Solv BBS-2.
[6] Photo activity cage [61-cm (24-in.) diameter and 38-cm (15-in.) high cylinder], Lehigh Valley Electronics, Inc., Lehigh Valley, PA 18001

Table I—Sleeping Times of Female Rats Pretreated with Nicotinamide and Some Related Compounds[a]

Dose, mg/kg	Nicotinamide	3-Acetylpyridine	3-(1-Hydroxyethyl)pyridine
900	$17 \pm 2.5^{0.05}$	—[b]	—[b]
90	22 ± 3.2	—[b]	—[b]
45	29 ± 2.7	30 ± 1.8	26 ± 3.1
11	21 ± 3.1	30 ± 2.2	28 ± 0.4
45[c]	$40 \pm 5.2^{0.01}$	33 ± 3.5	$37 \pm 2.7^{0.05}$
45[c]		Nicotinic acid $34 \pm 3.2^{0.05}$	

[a] All drugs were administered intraperitoneally in aqueous solutions. The dose of hexobarbital was 50 mg/kg. Sleeping times are tabulated as mean ± *SE* in minutes. Statistical significance is indicated by superscripts, and the data were analyzed according to the *t* test. [b] Results were not obtainable at these doses due to deaths and the severe toxic signs during the 4 days of the experiments. [c] These data were obtained from acute experiments wherein the test substances were administered and 30 min later hexobarbital was also administered. Pretreatment at the other doses was carried out for 3 days prior to the administration of hexobarbital.

Table II—Relationships between Toxicity, Coenzyme Depletion, Gross Motor Activity, and Sleeping Time in the Mouse

Compound	Dose[a], mg/kg	Urinary-^{14}C[b], % Increase	Motor Activity[c], % Decrease	Sleeping Time[d], min
Methylnicotinate	450	300	42	60
3-Methylpyridine	250	290	74	139
3-Pyridylcarbinol	1500	260	93	248
Nicotinic acid	3090	190	89	203
Pyridine-3-carboxaldehyde	720	120	92	225
Methyl-3-pyridylcarbinol	240	52	68	287
Nicotinamide	1940	42	95	71
Pyrazinamide	705	40	92	86
Salicylic acid	500	27	46	32
3-Acetylpyridine	182	0	0	89
Isoniazid	103	0	16	181
Aspirin	800	0	49	34
p-Aminosalicylic acid	1800	0	28	34
Phenylbutazone	210	−25	73	79

[a] Doses are equal to the LD_{25}. [b] Shown as percentage increase above control, except for phenylbutazone, during the 16 hr after administration of the indicated compound. [c] Shown as the mean percentage decrease from control with two groups of five mice each in all comparisons. The maximal variability in the controls was ±15%. [d] Control values were equal to 32 and 34 min in the groups of 10 mice.

dose, an apparent mild sedation was noted, with return to normal activity within 20 min. During 10–24 hr after the administration of these latter two compounds, all signs discussed were seen in some mice, with deaths occurring throughout the 7-day period as described previously (20, 21).

Induced Excretion of ^{14}C—The quantitative results of the induced urinary excretion of radioactivity from the ^{14}C-labeled mice are shown in Tables II and III. The mean (429,000 dpm) of the induced ^{14}C-excretion values for the first six compounds in Table III is significantly different ($p < 0.001$, Student *t* test) from the control mean (310,000 dpm), even though one of five animals died in each of three of the four experiments. Similar data with these and some additional compounds are shown in Table II. The induced excretions of ^{14}C by nicotinic acid, 3-pyridylcarbinol, 3-methylpyridine, and methylnicotinate are much greater than the control increment. These studies indicate that 12 of the 17 compounds shown in the two tables at doses equal to their LD_{25}'s significantly altered the normal excretion of nicotinamide and/or its metabolites in the urine of the mouse.

Patterns of Urinary ^{14}C from 7-^{14}C-Nicotinic Acid-Labeled Mice—The information on the excretion of radioactive metabolites of nicotinic acid (Table IV) extends the preliminary studies of Roth *et al.* (15) and Lin and Johnson (22). The values show that less than 10% of the ^{14}C in the urine is 7-^{14}C-nicotinic acid. The control values for percent of the urinary ^{14}C excreted as 1-methylnicotinamide and pyridones show very little variation[7]. However, the values for nicotinic acid and nicotinamide in the control experiments and all four parameters in the treated groups show wide variations. Thus, comparisons between the control and treated groups can best be made in regard to percent ^{14}C-1-methylnicotinamide and percent ^{14}C-pyridones.

[7] The urinary radioactivity corresponding chromatographically to 1-methylnicotinamide (1-methyl-3-carbamoylpyridinium ion) was shown by the isotope dilution technique in similar experiments (17) to be at least 84% accountable as 1-^{14}C-methylnicotinamide. Similarly, 1-methyl-3-carbamoyl-6-pyridone was 93% accountable as the 1-^{14}C-methyl-3-carbamoyl-6-pyridone and 1-methyl-3-carbamoyl-4-pyridone was 98% accountable *via* isolation as the 1-^{14}C-methyl-3-carbamoyl-4-pyridone *N*-oxide.

Five of the 11 compounds show percentages significantly different from the control means, thus indicating an altered disposition of endogenously generated free nicotinamide in the mouse. Furthermore, these effects upon 7-^{14}C-nicotinamide disposition are limited to compounds having significant effects upon total urinary-^{14}C except with the pH 4 buffer.

Tissue Distribution of ^{14}C—The relative ratios of the tissue specific activities for kidneys, lungs, brain, and liver are 1.0, 1.4, 3.1, and 4.2, respectively, and can be calculated from the values in Table V. Of the four tissue sites, pyridine-3-carboxaldehyde alone

Table III—Toxicity and ^{14}C-Nicotinamide Depletion after the Intraperitoneal Administration of Nicotinamide and Some Analogous Compounds to the Female Mouse

Compound (Molecular Weight)	LD_{25}[a], mg/kg	$\frac{LD_{75}}{LD_{25}}$	Urinary-^{14}C Increment[b], dpm
Nicotinic acid (123)	3087	1.85	578,000 (5)
Nicotinamide (122)	1940	1.14	130,000 (4)
3-Cyanopyridine (104)	810	1.43	−110,000 (5)
Pyridine-3-carboxaldehyde (107)	720	1.49	366,000 (5)
Pyrazinamide (123)	705	1.37	125,000 (4)
3-(1-Hydroxyethyl)pyridine (121)	239	1.22	160,000 (5)
3-Acetylpyridine (121)	182	1.45	19,000[c] (5)
Thionicotinamide (138)	105	3.42	80,000[c] (4)
Isoniazid (137)	103	1.38	3,000[c] (5)
Nicotine (162)	6.2	1.69	13,000[c] (5)

[a] The LD values were calculated according to the method of Litchfield and Wilcoxon with five or six groups, each containing 10–30 mice. A 7-day observation period was used for determining survival or death. [b] These values indicate the differences between the control mean ± *SD* (310,000 ± 43,000 dpm) from eight experiments and the experimentally obtained values for the number of mice (parentheses) that survived the 16-hr experiments. The control and treated groups each initially contained five mice in the ^{14}C-excretion studies. [c] These values do not differ significantly from the control mean; *i.e.*, they equal less than 2 *SD*.

Table IV—Percentages of Radioactivity Corresponding to Known Metabolites of Nicotinamide in the Urine of ^{14}C-Labeled Mice Treated with Nicotinamide and Some Related Compounds[a]

Percentage of Total Urinary Radioactivity

Control

Urinary-^{14}C Constituents											Mean ± *SD*
^{14}C-1-Methylnicotinamide	37	34	32	36	29	26	35	26	36		32 ± 4.6
^{14}C-Nicotinic acid	17	—	5.7	15	10	5.6	7.8	8.1	4.4		7.4 ± 5.0
^{14}C-Pyridones	38	53	53	41	40	47	55	41	46		45 ± 6.3
^{14}C-Nicotinamide	6.4	7.9	7.4	8.4	16	21	2.1	25	14		12 ± 7.5

Treated

Urinary-^{14}C Constituents	Nicotinamide[b]	1-Methylnicotinamide	Nicotinic Acid[b]	3-Pyridylcarbinol[b]	3-Acetylpyridine	INH	Pyrazinamide[b]	Thionicotinamide[b]	3-Cyanopyridine[b]	3-PE[b]	NIC	HOH	Dimethyl Sulfoxide	pH 4	Mean ± *SD*
^{14}C-1-Methylnicotinamide	11[c]	41	36	50[c]	40	27	49[c]	62[c]	75[c]	35	25	32	33	33	—
^{14}C-Nicotinic acid	10	—	3.7	7.0	<1.0	6.3	5.5	4.1	<1.0	2.6	5.5	—	9.5	18[c]	—
^{14}C-Pyridones	<1.0[c]	48	51	41	35	52	29[c]	16[c]	10[c]	40	55	57	52	46	—
^{14}C-Nicotinamide	64[c]	6.8	8.4	2.0	25	15	16	18	11	22	14	11	5.2	4.7	—

[a] The percent values are reported as 1-methylnicotinamide, 1-methyl-3-carbamoyl-4-pyridone plus 1-methyl-3-carbamoyl-6-pyridone (pyridones), and nicotinamide due to the localization of the radioactivity on paper chromatograms at R_f values coincident with those for these known metabolites of nicotinic acid. Each value represents one group of five mice. [b] The administration of these compounds was shown to have caused a significant change in the total radioactivity excreted in the urine of the 7-^{14}C-nicotinic acid-pretreated mice. [c] These values are considered to be significantly different from the corresponding mean control values; *i.e.*, they differ by more than 2 *SD* from the mean.

had significant effects at three sites. Nicotinamide and 3-(1-hydroxyethyl)pyridine had an effect at one site (lungs).

Whereas few significant decreases in the tissue specific activities are seen after treatment with the nine compounds, of the 36 values shown, only seven are above their respective control means. This indicates that a general mobilization is occurring, especially in the liver where all of the treated values are lower than the control mean. The lack of any significant tissue depletion after nicotinic acid administration was especially surprising in view of the large total urinary-^{14}C excretion shown in Table III.

Hexobarbital Sleeping Times in Rats—Chronic (3-day pretreatment with single daily intraperitoneal administrations) effects with 3-acetylpyridine and 3-(1-hydroxyethyl)pyridine were not seen due to death and severe toxic signs at the elevated doses shown in Table I. It is comparatively shown, however, that 3-day pretreatment with nicotinamide, 3-acetylpyridine, and 3-(1-hydroxyethyl)pyridine had no effects upon sleeping times. In the acute situation where hexobarbital was administered 30 min rather than 24 hr after the compound, prolonged sleeping times were seen except with 3-acetylpyridine. This lack of an effect is in agreement with the results shown in Tables II–V which also show no significant effects of 3-acetylpyridine upon processes involving the nicotinamide dinucleotides.

Motor Activity, Hexobarbital Sleeping Time, and Toxicity Correlates in Mice—As described, many compounds apparently induced a general central nervous system depression in the mice. The decreases in gross motor activities and, perhaps, the increases in hexobarbital sleeping times (Table II) support these initial observations. Thus, the altered coenzyme turnover, as shown by the tabulated changes in urinary ^{14}C, was accompanied by decreased motor activities except with aspirin (acetylsalicylic acid) and, perhaps, *p*-aminosalicylic acid since values of about 15% are within the variability exhibited in control groups.

The data in Table II also suggest a relationship between altered urinary-^{14}C (and thus nicotinamide dinucleotide turnover) and sleeping time since compounds that showed low or no alteration of urinary-^{14}C had small or no measurable effects upon the sleeping time at the indicated doses. The first eight compounds in Table II showed significant alterations in coenzyme turnover, motor activity, and sleeping time. Phenylbutazone was the only other drug that similarly altered all three parameters.

DISCUSSION

Nicotinic Acid Site—The results in Tables III and IV can be analyzed in terms of two sites of action for seven of the 10 substances. The largest increases in urinary-^{14}C excretion (Table III) were obtained with nicotinic acid and pyridine-3-carboxaldehyde. The result with nicotinic acid is interpretable as an increased synthesis and resultant turnover of NAD, with the consequent liberation of 7-^{14}C-nicotinamide as per the biosynthetic pathway (Scheme I). This normal route to NAD biosynthesis will be referred to as the nicotinic acid site of action and was recently discussed (23).

As shown in Table IV, after nicotinic acid treatment, the liberated 7-^{14}C-nicotinamide is metabolized and excreted in the normal but accelerated manner since no differences in the relative percentages between the control and treated values are apparent. The effects of pyridine-3-carboxaldehyde can be similarly explained since it may be metabolized to nicotinic acid in a manner analogous to the known oxidations of aldehydes to their corresponding acids. However, the administration of pyridine-3-carboxaldehyde results in an elevated level of ^{14}C-1-methylnicotinamide in the urine (Table IV). This indicates that pyridine-3-carboxaldehyde can alter the normal disposition of nicotinamide, perhaps competitively, in agreement with a possible action also at the nicotinamide site to be described. The lack of any obvious relationship between the toxicity and ^{14}C-depletion with 3-acetylpyridine, isoniazid, and nicotine indicates that these three substances act predominantly at other than the nicotinamide dinucleotides to exert their lethal effects.

Nicotinamide Site—Treatment with nicotinamide resulted in a significant increase in urinary-^{14}C and a considerably altered disposition of endogenously liberated 7-^{14}C-nicotinamide. Thus, the 7-^{14}C-nicotinamide entered the nicotinamide pool with the result that little methylation of the ^{14}C-nicotinamide occurred; 64%

Table V—Tissue-Specific Activities in the Mouse 40 hr after Administration of 7-^{14}C-Nicotinic Acid and the Effects of Treatment with LD_{25} Doses of Nicotinic Acid and Related Compounds

Compound	Tissue Radioactivity × 10^{-3}, dpm/g			
	Brain	Lungs	Liver	Kidneys
Control[a]				
	6.01 ± 1.43	13.0 ± 1.99	4.46 ± 0.94	18.72 ± 6.96
Treated				
Isoniazid	6.76	10.08	3.03	21.30
Pyridine-3-carboxaldehyde[b]	2.64[c]	4.66[c]	2.16[c]	12.56
3-Cyanopyridine[b]	4.87	12.40	2.87	11.90
Nicotinic acid[b]	5.66	12.30	2.78	12.49
Thionicotinamide[b]	7.06	14.12	3.40	18.00
Pyrazinamide[b]	5.62	15.35	2.84	20.35
3-Acetylpyridine	3.66	15.15	2.66	15.59
Nicotinamide[b]	5.25	8.53[c]	4.27	10.35
3-(1-Hydroxyethyl)pyridine[b]	3.50	7.50[c]	3.14	10.40

[a] Each control value is the mean of five control groups of five mice each ± *SD*, and each treated value is the pooled value from five mice in one treatment group. [b] These compounds caused significant alterations in 16-hr urinary excretion of ^{14}C when administered 24 hr after 7-^{14}C-nicotinic acid. [c] These values differ by more than 2 *SD* from their respective control means.

of the urinary-^{14}C was accountable as ^{14}C-nicotinamide and only 11% as ^{14}C-1-methylnicotinamide compared to the control means of 6% ^{14}C-nicotinamide and 37% ^{14}C-1-methylnicotinamide. Furthermore, essentially no ^{14}C-pyridones were found in the urine.

These relative percentage values indicate that the normal route of nicotinamide metabolism from the NAD source is from NAD to nicotinamide to 1-methylnicotinamide to pyridones with little nicotinic acid as an intermediary since the values for ^{14}C-nicotinic acid are essentially unchanged. These results with nicotinamide can be interpreted as the consequence of a competition between the administered nicotinamide and endogenous 7-^{14}C-nicotinamide at the level of glycohydrolase (EC 3.2.2.6, Scheme II), involving 7-^{14}C-nicotinamide-labeled endogenous NAD and the endogenous nicotinamide pool (7, 24, 25). This site of action at the glycohydrolase will be referred to as the nicotinamide site.

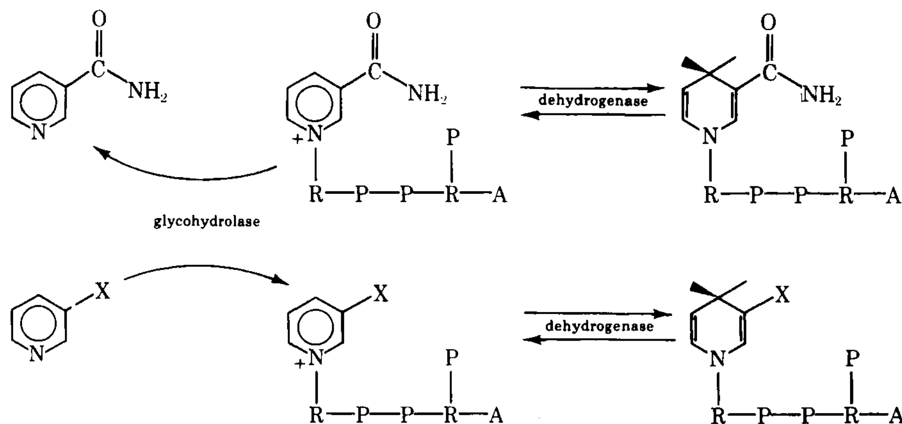
Nucleotide Source of Urinary-^{14}C—Although the effects of nicotinamide on urinary-^{14}C excretion may be interpreted as due to an action at the nicotinic acid site *via* the hydrolysis of nicotinamide to nicotinic acid, a consideration of the results with pyrazinamide, thionicotinamide, and 3-(1-hydroxyethyl)pyridine makes the nicotinamide site more probable as the major site of action of these four compounds, since the experiments afforded similar and significant effects upon urinary-^{14}C excretion (Tables III and IV). Additionally, in view of the structural differences between these compounds and nicotinic acid, it seems unlikely that either they or their metabolites could participate in the necessary enzymatic reactions in the biosynthetic pathway to NAD, thus causing a liberation of 7-^{14}C-nicotinamide.

Moreover, in view of the structural similarities especially among the three amides, it seems more plausible that a common site of action may be only at glycohydrolase. A further similarity of action among nicotinamide, pyrazinamide, and thionicotinamide is indicated by their alterations of the normal disposition of 7-^{14}C-nicotinamide (Table IV).

The increased excretion of urinary-^{14}C is perhaps the result of washing out a circulating pool of 7-^{14}C-nicotinamide and its metabolites. However, results with 1-methylnicotinamide (Table IV) argue against this possible site of action. 1-Methylnicotinamide caused neither an increased urinary-^{14}C nor an altered 7-^{14}C-nicotinamide metabolite pattern in the urine. Since the administered 1-methylnicotinamide and its pyridone metabolites would have depleted any circulating 7-^{14}C-1-methylnicotinamide and 7-^{14}C-pyridones, one can conclude that a normal turnover of the 7-^{14}C-nicotinamide dinucleotides was maintained and that the slow and normal metabolism of 7-^{14}C-nicotinamide was not measurably altered by 1-methylnicotinamide, either directly or *via* feedback inhibition of NAD metabolism.

Decreased Nucleotide Turnover—The decreased rate of NAD turnover, shown in Tables II and III as a decreased urinary-^{14}C excretion, after 3-cyanopyridine and phenylbutazone administration may have several bases. Initially, 3-cyanopyridine may inhibit the normal functioning of glycohydrolase and thus reduce the rate of NAD turnover. Additionally, the 16-hr urine volume was 2.8 ml, the lowest volume recorded in a 2.8–21-ml range with the control mean of 8.5 ml.

The decreased urinary-^{14}C after phenylbutazone administration is, however, not interpretable in terms of the two possible sites of action of the pyridine compounds since the chemical structures are apparently unrelated. However, a general action at the enzyme level *via* allosteric effects is suggested in view of the known high affinity shown by phenylbutazone for serum albumin. This allosteric effect could lead to an increased binding of the coenzyme and,



Scheme II—Illustration of the nicotinamide site of competitive antimetabolite (3-X-pyridine) incorporation into the nicotinamide dinucleotide pool

thus, an altered turnover rate. This alternative to the nicotinic acid and nicotinamide sites of action is supported by the general depleting effects (Table V) and will be discussed.

Coenzyme Binding Site—In view of the correlation between the equitoxic doses and their effects on the disposition of endogenous 7-^{14}C-nicotinamide, a cause-and-effect relationship is suggested for 12 of the 17 compounds discussed. Since nicotinic acid and nicotinamide are included in this correlation, the mediation of the toxic signs *via* abnormal dinucleotides analogous to NAD and/or NADP is seemingly obviated.

Although the dinucleotide analogs may contribute to the toxic manifestations, their formation *in vivo* would also remove the nicotinamide antimetabolites from the steady-state competition with nicotinamide at the level of glycohydrolase (26). Sund (27) reasoned that the nicotinamide dinucleotides are essentially completely bound to their respective apoenzymes. Reduction in the degree of binding could lead to their metabolism, to increased turnover, and to increased urinary-^{14}C. The loss of the apoenzyme *via* metabolism would also be a likely consequence of coenzyme depletion. Thus, the described results could be interpreted in terms of a third site of action at the level of coenzyme binding. This action allows an explanation of all observed results with the pyridine compounds, salicylic acid, and phenylbutazone.

The effects of nicotinamide, nicotinic acid, and 3-(1-hydroxyethyl)pyridine upon hexobarbital-induced sleeping times in the rat (Table I) support and extend the evidence for enzyme-related interactions of nicotinamide and other compounds that alter biological functions. An allosteric effect upon microsomal drug-metabolizing enzymes has also been reported with nicotinamide (28) and with metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) (29).

Tissue Site of Nucleotide Depletion—It has been reported (7) that the administration of 3-acetylpyridine to the mouse resulted in increased levels of liver NAD which were, however, less than those caused by nicotinamide but more than those caused by nicotinic acid. The present results show that 3-acetylpyridine was ineffective in altering the normal disposition of 7-^{14}C-nicotinamide. Since the normal pattern of metabolites of nicotinamide was obtained after 3-acetylpyridine administration, the metabolism of even these small amounts of ^{14}C-nicotinamide and ^{14}C-1-methyl nicotinamide was not altered by 3-acetylpyridine.

In contrast to the hypothesis of Herken (25), the toxicity of 3-acetylpyridine is, therefore, not likely related to interference in the normal synthesis or breakdown of NAD. Furthermore, based upon the normal urinary-^{14}C excretion after 3-acetylpyridine administration, the toxicologically significant existence of 3-acetylpyridine analogs of the nicotinamide dinucleotides seems unlikely. This failure to obtain any significant effect upon the disposition of ^{14}C-nicotinamide after 3-acetylpyridine administration argues against any overall nicotinamide-depleting effect by 3-acetylpyridine. However, a highly specific competitive interaction of 3-acetylpyridine and nicotinamide in a small vital area of the brain could have gone undetected (25, 30). An explanation of the so-called increases in liver NAD after treatment with 3-acetylpyridine would seem to require the existence of a unique substance very similar in chemical and spectral properties to NAD, *i.e.*, the "apparent liver pyridine nucleotide" suggested by Hayman *et al.* (8).

The distinct differences between the results with 3-acetylpyridine and 3-(1-hydroxyethyl)pyridine are entirely unexpected. Previous experiments (21) with mice and rats showed that the LD_{50} values for both compounds were essentially equal, and these results were confirmed by tabulated LD_{25} values in this study. Furthermore, the toxic signs and time courses of these signs were indistinguishable, whereas the onset times differed by 1 hr in previous experiments (21, 24, 31, 32). However, both 3-acetylpyridine and 3-(1-hydroxyethyl)pyridine caused similar increases in "apparent" urinary 1-methylnicotinamide and nicotinic acid in studies in the dog (33).

Again, a possible explanation of these different results may be that 3-(1-hydroxyethyl)pyridine has a more general and 3-acetylpyridine a more specific depleting effect upon the nicotinamide-containing dinucleotides. This possibility is supported by the work on depletion of tissue-^{14}C shown in Table V. The apparent site specificity shown by pyridine-3-carboxaldehyde, nicotinamide, and 3-(1-hydroxyethyl)pyridine could be extended to other levels of biological organization. Thus, depending upon the sites of depletion, either a toxic or, perhaps, therapeutic effect could be obtained.

Toxic *versus* Therapeutic Effects of Coenzyme Depletion—Nicotinic acid, 3-pyridylcarbinol, phenylbutazone, and the salicylates are therapeutically useful agents. The first two drugs are used for their vasodilator effects. The site(s) of the ^{14}C-depletion by nicotinic acid did not include the brain, kidneys, liver, and lungs (Table V). The vascular site was not investigated, but these studies are in progress with the vasodilator substances. Thus, site-specific coenzyme depletion could be a basis of toxic and/or therapeutic effects, depending upon the binding energies of the various coenzyme–apoenzyme complexes (34) and the relative affinities of the agents for the various tissues, as shown by Chang (35) with the coenzyme-depleting agent streptozotocin.

SUMMARY AND CONCLUSIONS

This investigation was initiated to ascertain a biochemical parameter that correlated with an acute toxic effect. The toxic effects of 17 compounds were studied in relation to induced changes in the turnover of coenzymes (nicotinamide adenine dinucleotides), nicotinamide metabolism, hexobarbital sleeping time, and gross motor activity in the mouse. Twelve of the compounds altered the turnover of the ^{14}C-labeled nicotinamide dinucleotide pool in the mouse, with a resultant general depression of the animals.

The increased dinucleotide turnover was discussed in terms of drug action at a nicotinic acid site, a nicotinamide site, and coenzyme binding site. The first two sites allow the possible incorporation of nicotinamide analogs into the organism and the formation of analogs of the dinucleotides, but they also serve as routes for the inactivation of these pyridine compounds. The coenzyme binding site of action allowed the best explanation of the results. It is suggested that coenzyme depletion is a more general basis of the actions of these drugs and toxicants. Thus, coenzyme depletion and concomitant metabolism of the corresponding apoenzymes may be a basic mechanism of drug action leading to toxic and/or therapeutic effects.

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