

Effects of 6-Diazo-5-oxo-L-norleucine and Other Tumor Inhibitors on the Biosynthesis of Nicotinamide Adenine Dinucleotide in Mice¹

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Summary

NAD² in extracts of mouse liver and tumor was determined enzymically by ADH and chemically by reaction with cyanide. The presence of Sarcoma 180 caused a decrease in the level of NAD in the host liver. Of the 16 antitumor agents tested for inhibition of nicotinamide-induced biosynthesis of NAD, DON was the most effective. 6-Fluoronicotinamide, 5-fluoronicotinic acid, azaserine, and 6-mercaptapurine were inhibitory but less effective, and mitomycin, actinomycin, and amethopterin were ineffective. DON caused a slight decrease in the normal levels of liver and tumor NAD and completely prevented the increased synthesis of NAD induced by nicotinamide. Glutamine given before administration of DON and nicotinamide did not prevent the effect of DON. An accumulation of desamido-NAD in the livers of mice treated with nicotinamide plus DON was found approximately equal to the amounts of NAD synthesized in the absence of DON. It is suggested that DON inhibits the enzyme system that converts desamido-NAD to NAD—NAD synthetase.

Introduction

Previous studies from this laboratory (1-4) on the mechanism of action of tumor growth-inhibitors have been focused on effects on nucleic acid metabolism and, in greater detail, on biosynthesis and utilization of the constituent purine and pyrimidine bases. However, altered biosynthesis of purines, for instance, does not of itself explain the full action of an inhibitor.

To relate biosynthetic inhibition of purines more closely to growth inhibition, we became interested in the metabolism of the important adenine-containing coenzyme, NAD, which is so vital to cell growth. The effects of various substances on NAD biosynthesis and, in particular, the effect of the tumor inhibitor DON are reported here.

Materials and Methods

MATERIALS. For most experiments Swiss white mice (Carworth Farms) were used with or without Sarcoma 180 (S-180) trans-

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² The abbreviations used are: NAD, nicotinamide adenine dinucleotide; ADH, alcohol dehydrogenase; DON, 6-diazo-5-oxo-L-norleucine; desamido-NAD, nicotinic acid analog of NAD; TCA, trichloroacetic acid; NAm, nicotinamide; 6-AN, 6-aminonicotinamide; NMN, nicotinamide mononucleotide; NAc, nicotinic acid; and AMP, adenine monophosphate.

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plants. Experiments recorded in Chart 3 were performed on C57BL mice carrying Carcinoma 755 (Ca-755).

The 16 compounds tried for inhibitory effects on NAD biosynthesis were from the files of compounds submitted to the Sloan-Kettering Institute for Cancer Research for chemotherapeutic tests. Sources are acknowledged in the footnote to Table 2.

Nicotinamide-7-¹⁴C, specific activity 3 $\mu\text{c}/\mu\text{mole}$, was purchased from the New England Nuclear Corporation. A sample of desamido-NAD was kindly furnished by N. O. Kaplan. Solid crystalline alcohol dehydrogenase was obtained from the Worthington Biochemical Company; 1 mg was dissolved in 5 ml of phosphate buffer, 0.02 M, pH 7.5. This solution was not kept more than 2 days, and was constantly refrigerated.

METHODS. Test compounds were administered in single doses at or near the maximum tolerated dose. The liver (and tumors when present) of mice killed by cervical dislocation were excised rapidly. Extracts, completed within 3-4 min after sacrifice, were made either by the method of Jedeikin and Weinhouse (11) (0.05 M phosphate buffer, pH 5.4, in boiling water 1 min, homogenized 30 sec); or by homogenization at 4°C with 4 volumes of 0.6 N perchloric acid. The 1st method was used predominantly (see Table 1) when NAD levels were desired, and the 2nd method was used for subsequent column chromatography.

NAD was determined enzymatically with yeast alcohol dehydrogenase (5). Concurrently, routine readings of the cyanide-addition reaction were taken at 325 and 340 m μ (6). These values, although calculated as NAD, can properly be regarded as a measure of total oxidized pyridine nucleotides.

Results

In addition to the 2 methods of liver extraction cited above, 5% TCA was also tried (see, for instance, Ref. 14). From the comparison shown in Table 1, it seemed advisable to use Jedeikin and Weinhouse's buffer extract for routine determination of NAD.

Chart 1 shows the effect of tumor transplantation and growth (S-180) upon NAD levels in the host liver and in the sarcoma. An initial drop in the liver was partially overcome, followed by a gradual lowering. Sixteen days after transplantation, the presence of the tumor had affected the liver, which was much smaller than normal, and the NAD level was reduced about 70%.

Single injections of many inhibitors, alone, at the maximum tolerated dose did not markedly affect levels of NAD in liver or tumor within 24 hr. However, when observed over longer periods, DON (Chart 2) gradually lowered NAD in liver for 96 hr to about 75% of starting level, after which recovery followed.

TABLE 1
RECOVERY OF NAD FROM NORMAL MOUSE LIVER BY DIFFERENT
EXTRACTANTS^a

Extractant	μg NAD/gm liver
1. 4 ml. of phosphate buffer (pH 5.4)/gm liver (108)	556 \pm 58
2. 4 ml of 0.6 N HClO ₄ /gm liver (9)	414 \pm 35
3. 4 ml of 5% TCA/gm liver ^b (9)	432 \pm 43

^a Preparations for No. 1 were centrifuged as in Ref. 11; for No. 2, they were neutralized with KOH and centrifuged; for No. 3, they were neutralized with NaOH. The total number of mice used are in parentheses. Although No. 1 includes many experiments, the first experiment, with 6 mice, gave an average NAD (nicotinamide adenine dinucleotide) value of 571 $\mu\text{g}/\text{gm}$.

^b TCA, trichloroacetic acid.

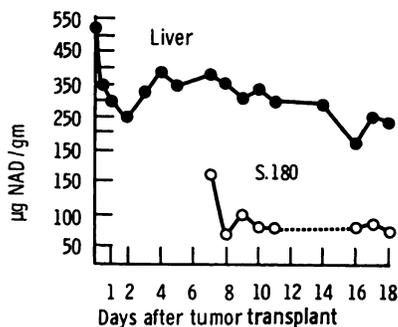


CHART 1. NAD content in liver and developing tumor (S-180) in Swiss white mice. Each point represents the average of 3 animals.

It is well known that an injection of a large dose of NAM into mice stimulates biosynthesis of NAD to a temporary, but very large, value (12). By administering test compounds concurrently with NAM, the effects of some of these substances on NAD biosynthesis can be observed. For example, Chart 3 shows the effects of NAM and 6-AN plus NAM on NAD biosynthesis in Ca-755 and the livers of C57BL mice. 6-AN decreased NAD biosynthesis by about 50%. The chemotherapeutic tests carried out in this Institute by D. A. Clarke (unpublished data) also indicated that 6-AN caused about 50% inhibition of Ca-755.

Table 2 shows the effects of 16 "tumor inhibitors" upon NAM-induced NAD biosynthesis in normal Swiss white mice; for convenience, all were sacrificed 5 hr after injection. 6-AN was ineffective in this experiment, as it was against S-180 growth, according to the chemotherapeutic tests of Clarke. Of interest in this Table were DON and the DON-containing peptide, alazopeptin.

The effect of DON on NAD biosynthesis is shown in more detail in Chart 4. Almost a complete inhibition of NAM-induced biosynthesis was seen when DON was given concurrently with NAM. Since DON is a potent glutamine antagonist (15), the effect of adding glutamine was also determined. Glutamine, 500 mg/kg, was given 3 times: at 24 hr, at 12 hr, and immediately prior to NAM plus DON. Surprisingly, the imposition of glutamine lowered NAD biosynthesis. The mice were obviously sick, and the livers were smaller than in the NAM plus DON group.

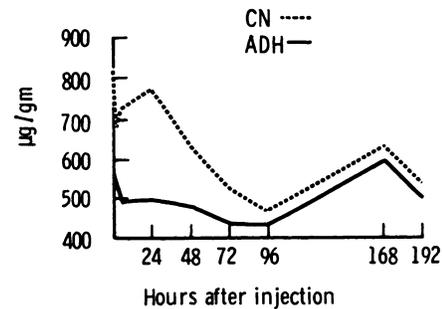


CHART 2. Effect of a single dose of DON (50 mg/kg) on mouse liver NAD level. Each point represents the average of 6 normal animals. ADH, NAD level as determined enzymically with alcohol dehydrogenase; CN, cyanide reaction, calculated as NAD.

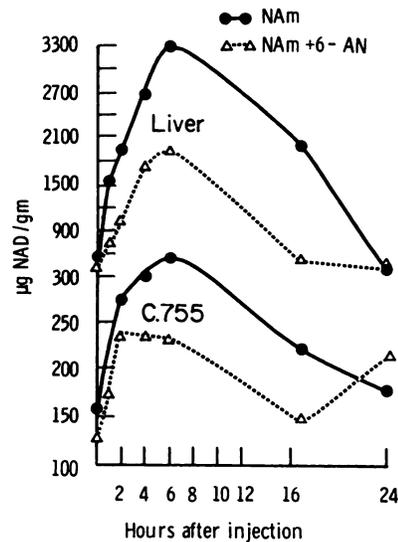


CHART 3. Effect of 6-AN on NAM-induced synthesis of NAD in C57BL mice bearing Ca-755. Upper curve, levels in liver; lower, levels in tumor. Each point represents the average of 3 animals.

Though not shown, NAM plus glutamine gave the same results as NAM alone, with no obvious ill effects to the mice.

Concurrent cyanide-reactive values for NAM plus DON, which were determined in the experiment illustrated in Chart 4, corresponded closely to the NAM curve. Apparently, though DON prevented the synthesis of NAD, a pyridine-containing compound was being "accumulated" at about the level that NAD would have attained had DON not been given. Table 3 illustrates this point and shows the approximate duration of this "DON effect." By 96 hr the 1 injection of DON had almost lost its effect on NAD synthesis, and the level of cyanide-reacting substance had almost returned to normal.

To determine the identity of the cyanide-reacting compound being accumulated, NAM-7-¹⁴C (3 $\mu\text{c}/\mu\text{mole}$, 1 $\mu\text{c}/\text{mouse}$ injected) plus carrier NAM (to give 500 mg/kg) and DON were injected into 10 normal mice. Extracts of the livers made with perchloric acid 5 hr after injection were pooled.

The unfractionated extract was spotted on Whatman No. 1 filter paper and subjected to descending chromatography in 70% ethanol-30% 1 M ammonium acetate, pH 5, which is a solvent system reported to "separate adequately" NAD and desamido-

TABLE 2
EFFECT OF VARIOUS TUMOR INHIBITORS UPON NAM-INDUCED NAD BIOSYNTHESIS IN NORMAL SWISS MICE^a

Drug ^b	NAm ^c (μg NAD/gm)	NAm + drug (μg NAD/gm)	% Change
Mitomycin C	3600	4020	+11.7
6-Aminonicotinamide	2870	2980	
Triethylenemelamine	3325	3290	
Actinomycin C	3515	3590	
Amethopterin	3170	2980	
5-Fluoronicotinamide	3060	2835	
5-Aminonicotinamide	3420	3610	
2-Ethylaminothiadiazole	3590	2845	-21
4-Aminoimidazo[4,5-d]pyridazine	3260	2520	-23
Glutamic acid γ-hydrazide	2950	2120	-28
6-Mercaptopurine	2870	1910	-33
Azaserine	2583	1703	-34
6-Fluoronicotinamide	3025	1785	-41
5-Fluoronicotinic acid	3355	1760	-48
6-Diazo-5-oxo-L-norleucine	2860	770	-73
Alazopeptin	2837	583	-79

^a All experimental values were determined 5 hr after injection. Abbreviations used are: NAm, nicotinamide; and NAD, nicotinamide adenine dinucleotide.

^b Sources: Mitomycin C and actinomycin C, Merck & Co.; 6-aminonicotinamide, Squibb Institute for Medical Research; 5-fluoronicotinamide, 5-aminonicotinamide, 6-fluoronicotinamide, and 5-fluoronicotinic acid, Eli Lilly; triethylenemelamine, American Cyanamid; amethopterin, 2-ethylaminothiadiazole, and alazopeptin, Lederle Laboratories; 4-aminoimidazo[4,5-d]pyridazine, Abbott Laboratories; 6-mercaptopurine, Burroughs-Wellcome; glutamic acid γ-hydrazide, azaserine, and 6-diazo-5-oxo-L-norleucine, Parke, Davis & Co.

^c Average of over 100 different experiments without drug: 3047 μg NAD/gm liver.

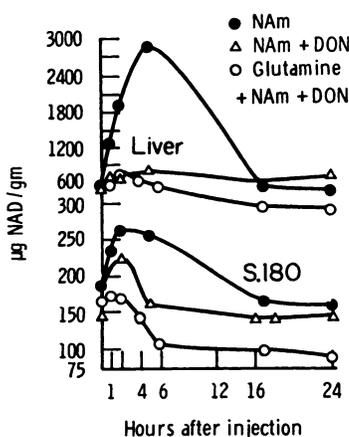


CHART 4. Effects of DON, and glutamine plus DON, on NAM-induced synthesis of NAD in Swiss white mice bearing S-180. Upper, levels in liver; lower, levels in tumor. Each point represents the average of 4 animals.

NAD (17). Chart 5 shows the pattern obtained. The lane containing the extract was scanned in an Atomic Accessories strip scanner, and 88% of the activity applied to the paper was in the

TABLE 3
LENGTH OF TIME OF "DON EFFECT" ON NAD BIOSYNTHESIS^a

Compound	Liver		Tumor ^b NAD
	NAD (μg/gm)	CN-reacting (μg/gm)	
No injection	443	439	144
NAm, 500 mg/kg	1980	328	236
DON, 50 mg/kg	443	297	123
DON plus			
NAm, same time	702	1728	160
NAm, 24 hr later	867	523	181
NAm, 96 hr later	1630	608	204

^a Mice killed 4 hr after NAm injection. NAD determined by ADH reaction; "CN-reacting" determined by reaction with 1.2 M aqueous KCN, results calculated as NAD, and ADH values subtracted. Abbreviations used are: DON, 6-diazo-5-oxo-L-norleucine; NAD, nicotinamide adenine dinucleotide; NAm, nicotinamide; and ADH, alcohol dehydrogenase.

^b Sarcoma 180 in Swiss white mice.

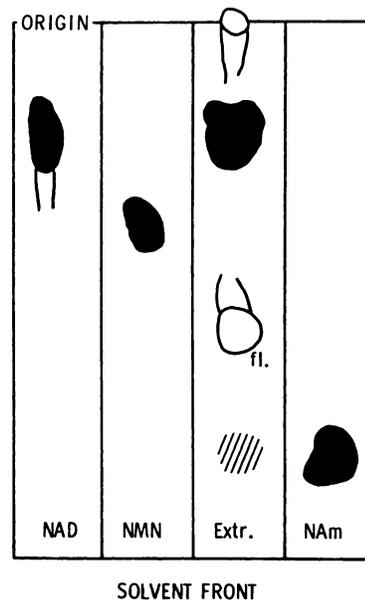


CHART 5. Paper chromatogram (drawing) of liver extract after injection of NAM-¹⁴C plus DON. Solvent system: 70% ethanol-30% 1 M ammonium acetate, pH 5 (17). Spots visualized under ultraviolet illumination.

spot on a level with NAD, 7% in the NAm spot, and about 2% at a level with NMN. The 2 fluorescent spots were not radioactive. The spot with the bulk of the activity, eluted from the paper with 0.1 N HCl, was a mixture of NAD (determined by ADH) and a cyanide-reacting substance.

The best method of separating the radioactive products proved to be ion-exchange chromatography on Dowex 1-X8, 200-400 mesh, in the formate form (9), as shown in Chart 6, with fractions eluted batchwise by increasing concentrations of formic acid.

From the control column (lower), 65% of the total radioactivity applied to the column was eluted in the NAD peak (III), 25% in the water "wash-out" (NAm), and 16% in desamido-

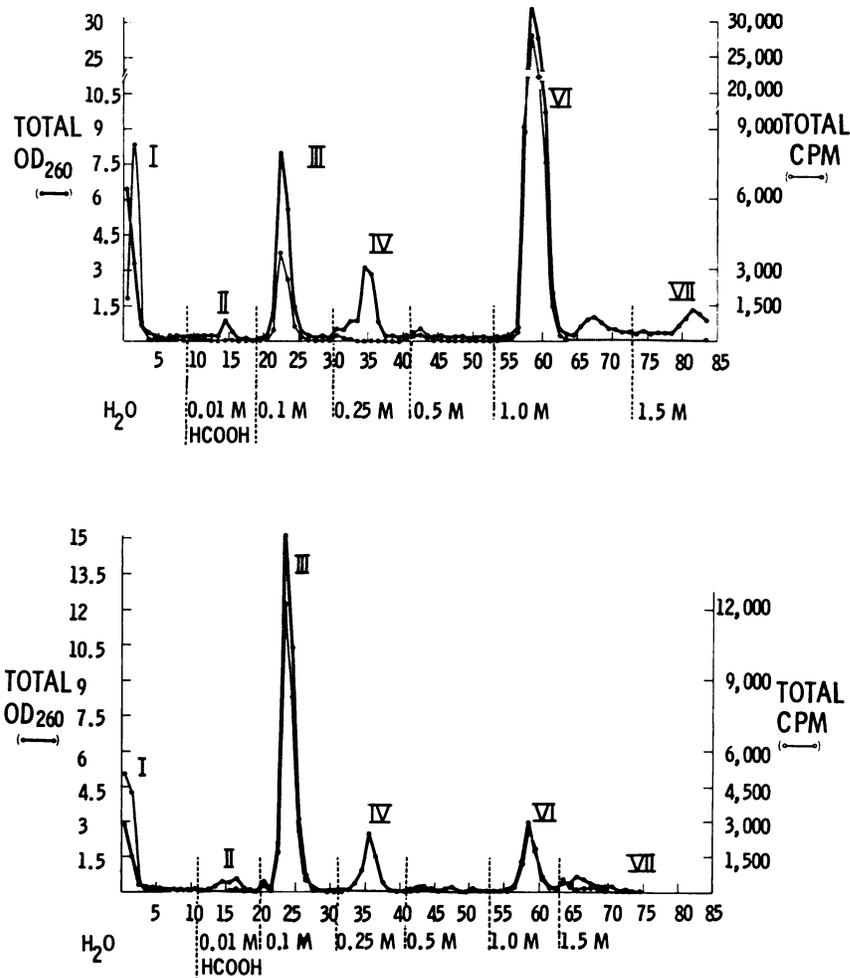


CHART 6. Column chromatography of perchloric acid extracts of liver of mice 5 hr after injection with DON plus $\text{NAm-}^{14}\text{C}$. Column: Dowex 1 formate; elution batchwise with formic acid as indicated. Peaks: I, mostly NAm; II, undetermined; III, NAD; IV, AMP; VI, desamido-NAD. Upper curve, DON plus $\text{NAm-}^{14}\text{C}$; lower, $\text{NAm-}^{14}\text{C}$ (control for upper). Put on column, upper: 5 ml, containing 116 μg of NAD/ml, 480 μg of cyanide-reacting substance/ml, 77,700 cpm; lower: 2 ml, containing 606 μg of NAD/ml, 688 μg of cyanide-reacting substance/ml, 38,920 cpm.

NAD (Peak VI). By contrast, the column on which the liver extract from DON-treated mice was fractionated (upper) resulted in only 8% of the total radioactivity in the NAD, 10% in NAm, and 80% in desamido-NAD. Peak VI was identified as desamido-NAD by co-chromatography and paper electrophoresis of this isolated substance with known desamido-NAD (see "Materials," above).

Table 4 records the specific activities and amounts of NAm, NAD, and desamido-NAD isolated by the column chromatography shown in Chart 6. There was a 78% lowering in the level of NAD with almost 50% less radioactivity. Although the desamido-NAD increased almost 6-fold, the specific radioactivity did not increase at all, indicating no appreciable inhibition by DON of steps prior to desamido-NAD in the biosynthetic sequence.

The level of desamido-NAD in mouse liver frequently approached 4000 $\mu\text{g}/\text{gm}$ of liver 5 hr after a single injection of 500 mg/kg of NAm and 50 mg/kg of DON.

Discussion

It is of interest that extraction of liver by acid gave over 20% less NAD than extraction by buffer (Table 1). Whether this is due to slight instability of NAD at low pH or incomplete solubility of the dinucleotide was not determined. In any event, comparisons should be made only if the same extractant has been used.

Waravdekar and associates (21, 22) reported that the presence of a tumor (Sarcoma 37) reduces the ability of blood and tissue homogenates from that tumor-bearing mouse to synthesize NAD directly from NMN *in vitro* (13). Our results (Chart 1) may agree with these observations, although it is debatable (17, 18) whether NMN is an actual intermediate in the biosynthesis of NAD from NAm. Shuster *et al.* (20) reported that "the growth of a number of solid tumors was found to have little effect on diphosphopyridine nucleotide synthesis in the liver." It is not clearly stated,

TABLE 4
SPECIFIC ACTIVITIES AND AMOUNTS OF ISOLATED COMPOUNDS FROM
THE 2 COLUMNS

COMPOUND	cpm/ μ mole		μ mole/gm LIVER	
	Control	DON ^a	Control	DON
NAm (Peak I)	6,290	3,192	2.8	2.7
NAD (Peak III)	13,080	7,470	3.6	0.78
desamido-NAD (Peak VI)	15,425	12,859	0.75	4.2

^a Abbreviations used are: DON, 6-diazo-5-oxo-L-norleucine; NAm, nicotinamide; NAD, nicotinamide adenine dinucleotide; and desamido-NAD, nicotinic acid analog of NAD.

but they presumably refer to the induced stimulated synthesis *in vivo* of NAD caused by injection of NAm into tumor-bearing animals. This is corroborated by the data in Charts 3 and 4. The levels of NAD reached were the same, whether or not the mice were tumor bearing (Ca-755 or S-180).

In the tumor system that was inhibited by 6-AN (Ca-755), NAD biosynthesis was also inhibited, whereas in S-180, neither growth nor NAD biosynthesis was affected by this compound. Whereas it is not known whether the pathway of NAD biosynthesis is different in an "analog-sensitive" tumor than in an insensitive tumor, it is more likely that S-180 cannot incorporate 6-AN into a nonphysiologic analog of NAD as can Ca-755 (7).

The effects of DON on NAD biosynthesis can be accounted for by the known facts that (a) desamido-NAD is an intermediate in the biosynthesis of NAD, whether NAm or NAc is the precursor (14, 17); (b) the conversion of desamido-NAD to NAD requires glutamine (17), although ammonia may be utilized to a small extent (18); (c) DON and azaserine are potent glutamine antagonists (15). That Narrod *et al.* (16) found no significant accumulation of "nicotinic acid DPN" in mouse liver after azaserine administration may be because DON is about 40 times more potent than azaserine as a glutamine antagonist (15), or because there is an enzyme in mouse liver that destroys azaserine but not DON (10, 19). We, too, found, as did Narrod *et al.* (16), that glutamine did not reverse the effects of the glutamine antagonist. This probably was because DON and azaserine are irreversible inhibitors (8, 15). The adverse effect of the combination of glutamine, DON, and NAm upon the animal and upon NAD biosynthesis is unexplainable.

Acknowledgments

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