# Significance of Enzymatically Catalyzed Exchange Reactions in Chemotherapy

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IT has been previously reported that animal tissue DPNases can catalyze an exchange reaction between the nicotinamide moiety of DPN and compounds related to nicotinamide according to the equation (*1*)

$$NRPPRA + X \rightarrow XRPPRA + N.$$

The pyridine compounds that to date have been found to undergo exchange with nicotinamide are isonicotinamide (*2*), isonicotinic acid hydrazide (*2–4*), marsilid (the isopropyl derivative of isonicotinic acid hydrazide) (*2*), 3-acetyl pyridine (*5*) and ethyl nicotinate (*6*); the resulting corresponding DPN analogs have now all been isolated.

The fact that analogs of DPN can be formed by an exchange reaction *in vitro* suggests the possibility that antimetabolites of nicotinamide might exert their pharmacologic action through such a mechanism. Furthermore, if these reactions occur in the whole animal, they may be of significance in developing a new type of approach for the use of chemotherapeutic agents. This paper (*7*) is the result of a preliminary effort to make use of an understanding of enzymatic mechanisms toward the development of pharmacologically active compounds. Evidence is presented that exchange reactions occur *in vivo*. In addition, this report is concerned with the significance of enzymatically catalyzed exchange reactions in elucidating the toxic action of an antimetabolite such as 3-acetyl pyridine.

Woolley (*8*) has found that feeding 3-acetyl pyridine to mice will produce symptoms of nicotinic acid deficiency when the level of the vitamin in the diet is low. Addition of either nicotinic acid or nicotinamide to the diet relieved the animals from the symptoms induced by the acetyl pyridine. Table 1 summarizes the toxicity studies that we have carried out on mice. Intraperitoneal administration of 500 mg of compound per kilogram of body weight usually results in 100 percent mortality. Most of the deaths occur about 4 hr after the injection of acetyl pyridine. The primary signs of toxicity seem to result from disturbance of the central nervous system. Details of the toxic manifestations will be presented elsewhere (*9*).

Table 1 also shows that the simultaneous administration of nicotinamide from 250 to 1000 mg/kg affords marked protection against the lethal effects of acetyl pyridine. It is of interest to note also that administered DPN has a protective action. In contrast, nicotinic acid does not protect against toxicity. Tryptophan also appears to be without effect (*10, 11*).

The fact that nicotinamide can protect the animals against the toxicity of the antimetabolite, whereas the acid does not, is of considerable interest, particularly since nicotinic acid and nicotinamide are considered to be of equal value in the diet. The inability of nicotinic acid to protect appears to be related to the finding that tissue DPNases do not promote an exchange between the nicotinamide of DPN and free nicotinic acid. On the other hand, it has been found by the use of $C^{14}$-labeled nicotinamide that animal DPNases will catalyze an exchange between the bound nicotinamide and free nicotinamide (*12*). In Table 2, data are presented that show that nicotinamide inhibits the formation of the acetyl pyridine analog from DPN and acetyl pyridine in a mouse brain homogenate. Nicotinic acid, in contrast, has no such effect.

From the results given in Tables 1 and 2, it appears likely that the toxicity rendered by the injection

Table 1. Effect of metabolites on the lethal toxicity of 3-acetyl pyridine in mice.*

| Metabolite | Dose (mg/kg) | 3-Acetyl pyridine (500 mg/kg) Dead/total |
|---|---|---|
| Nicotinamide | 1000 | 0/12 |
| | 500 | 0/12 |
| | 250 | 0/12 |
| DPN | 1500 | 0/6 |
| | 1000 | 0/12 |
| | 500 | 1/12 |
| | 250 | 2/6 |
| Nicotinic acid | 1000 | 6/6 |
| | 500 | 6/6 |
| | 250 | 6/6 |
| Tryptophan (DL) | 1000 | 5/6 |
| | 500 | 6/6 |
| | 250 | 6/6 |
| Controls | No metabolite | 23/23 |

* Metabolite administered intraperitoneally immediately prior to the administration of 3-acetyl pyridine subcutaneously. Mice: C × DBA hybrid ♂ 8 to 10 wk old.

of 3-acetyl pyridine is related to the formation of the corresponding analog of DPN. The protection produced by nicotinamide *in vivo* is certainly closely correlated to the inhibition of synthesis of the analog *in vitro*. Although nicotinamide and nicotinic acid are interchangeable in the diet, these compounds are not interchangeable in combatting antimetabolites such as 3-acetyl pyridine. The ineffectiveness of nicotinic acid *in vivo* indicates that exchange reactions do occur *in vivo*, since if a synthetic system was involved it might be expected that the acid would protect because it can replace nicotinamide in the diet. It therefore seems of importance in studying the action of an antimetabolite to ascertain whether the antimetabolite acts by competing with the metabolite in a synthetic reaction (that is, the synthesis of DPN from nicotinamide) or in exchange reactions of the DPNase type.

Table 2 demonstrates that nicotinamide has a somewhat greater affinity for the mouse brain DPNase than does the 3-acetyl pyridine. This is manifested in the inhibition of analog synthesis by the use of a considerably lower nicotinamide level. A concentration of 0.01*M* nicotinamide is effective in inhibiting the analog formation 69 percent in the presence of 0.1*M* acetyl pyridine. Figure 1 shows the effect of nicotinamide on the median lethal dose ($LD_{50}$) of acetyl pyridine (*13, 14*). It may be observed that by increasing the amount of nicotinamide injected, a pronounced increase in the $LD_{50}$ takes place. The protective effect of nicotinamide reaches a maximum at a level of 250 to 500 mg/kg of body weight. Further increase in nicotinamide concentration results in a loss in protection against the toxicity of the acetyl pyridine. This deleterious effect of excess nicotinamide is apparently due to the nicotinamide itself, since the metabolite itself produces toxicity when administered at a level of 2000 mg/kg or more.

Nicotinamide, when administered up to a period of

Table 2. Effect of nicotinamide and nicotinic acid on acetyl pyridine analog formation in mouse brain. Reaction mixtures contained 0.32 micromoles DPN, 0.1 ml mouse brain homogenate, 0.1*M* 3-acetyl pyridine, 0.05*M* phosphate (*p*H 7.5), total volume 0.6 ml, temp. 37°C, incubation time, 35 min.

| Metabolite (mole) | 3-Acetyl pyridine analog of DPN formed* (μmole) | Inhibition of analog formation (%) |
|---|---|---|
| Nicotinamide | | |
| 0.0 | 0.202 | |
| .2 | .0 | 100 |
| .1 | .014 | 93 |
| .05 | .034 | 83 |
| .01 | .063 | 69 |
| .001 | .180 | 10 |
| Nicotinic acid | | |
| 0.1 | 0.192 | 4 |
| .01 | .210 | 0 |

* Analog determined by change in optical density at 400 mμ after addition of yeast alcohol dehydrogenase (*6*).

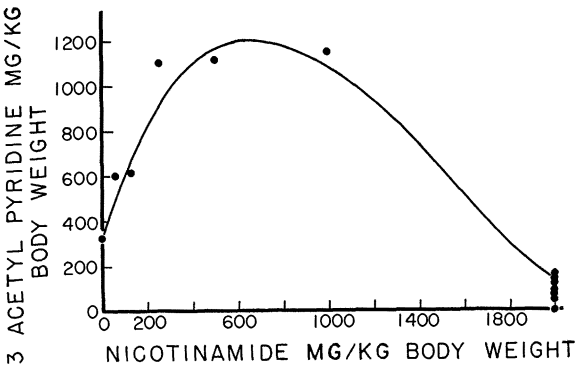


Fig. 1. Effect of nicotinamide on the median lethal dose ($LD_{50}$) of acetyl pyridine in mice.

2 to 3 hr after a lethal dose of acetyl pyridine is given, still has a protective influence. When the vitamin is given 4 hr prior to the antimetabolite, protection is again observed. If the nicotinamide, however, is administered more than 4 hr before the acetyl pyridine is introduced, a decrease in protection results. The fact that acetyl pyridine acts very rapidly, together with the characteristics of the antagonistic properties of nicotinamide, is certainly indicative that the lethal action of the compound is due to the exchange reaction catalyzed by the DPNase. Complete results of the time relationships of nicotinamide protection will be published elsewhere (*9*).

The dose-mortality response curve of 3-acetyl pyridine in mice is shown in Fig. 2. The $LD_{50} \pm 1$ S.E. is $305.5 \pm 12.5$ mg/kg. The slope of the line is 12.51 probits per tenfold increase in dose. Many deaths from acute toxicity occurred from 4 to 24 hr following administration of the antimetabolite. Additional deaths occurred as late as 6 days following drug administration. The animals in the latter group showed evidence of chronic toxicity, including anorexia and weight loss. Although it did not occur here, in other experiments treated animals occasionally did not succumb until 10 to 20 days following administration of the antimetabolite. At toxic levels of the antimetabolite, survivors of drug mortality showed evidence of anorexia and loss of weight. Acute toxicity occurs primarily at high doses of the drug. Chronic toxicity becomes more evident at doses just below the level of acute toxicity. The toxicity of acetyl pyridine when administered with nicotinamide is dependent upon the relative doses of antimetabolite and metabolite employed. Administration of appropriate doses of nicotinamide protects against both acute and chronic toxicity of acetyl pyridine.

The curve in Fig. 2 is quite steep. In fact, it appears to be twice as steep as similar plots obtained with 6-mercaptopurine or aminopterin (*15*). It is of interest to examine the significance of the curve particularly with respect to the fate of administered acetyl pyridine. It is known that acetyl pyridine can partially fulfill the nicotinamide or nicotinic acid requirement in the diet (*16*) It has also been found that administration of acetyl pyridine to animals re-

sults in the presence of nicotinamide and N-methyl nicotinamide in the urine (*17–19*). It thus appears likely that acetyl pyridine can be converted to either nicotinic acid or to its amide.

Further evidence confirming the conversion of acetyl pyridine to nicotinamide is presented in Table 3. Injection of acetyl pyridine to mice leads to a fourfold rise in the DPN level of the liver. Nicotinamide produces an eightfold increase in the liver coenzyme content. This exceptionally great increase in pyridine nucleotide content was quite unexpected (*20*). It is of interest to note that we have found that acetyl pyridine is a better precursor of DPN in the liver than nicotinic acid is. The mechanism by which acetyl pyridine is converted to nicotinamide bound in DPN is now under investigation in this laboratory.

Although acetyl pyridine does give rise to DPN, no acetyl pyridine analog was found in the liver. Hence it appears that the liver converts acetyl pyridine to nicotinamide and that a large amount of acetyl pyridine would be detoxified by such a conversion. However, the conversion of acetyl pyridine to free nicotinamide or nicotinamide bound in DPN does not occur in a number of other tissues. An investigation of extrahepatic tissues indicated that some analog was formed in the brain and in the spleen after injection of the acetyl pyridine. No increase in DPN occurred in these tissues after the introduction of the antimetabolite.

One explanation of the steepness of the curve in Fig. 2 might be that acetyl pyridine can be handled by the liver up to a certain amount, but after the liver becomes saturated with the compound the excess might then become incorporated as analog via the DPNase system in the extrahepatic tissues. The toxicity of acetyl pyridine may therefore be due to formation of the analog in the nervous system.

Acetyl pyridine is in a sense unique in that it is an antimetabolite that can be detoxified by its conversion to the metabolite. The compound is also of interest from a pharmacologic point of view, because its toxicity depends greatly on the differences in its reactivity in various tissues.

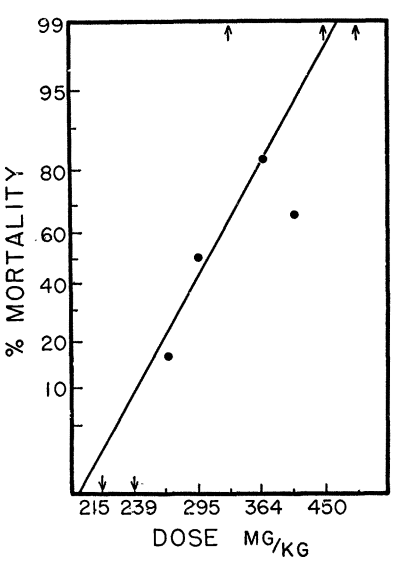


Fig. 2. Dose-mortality response curve of 3-acetyl pyridine in mice. 0 percent or 100 percent mortality represented by arrows.

Table 3. Effect of acetyl pyridine and nicotinamide injection on DPN* content of mouse liver. Animals were sacrificed 3 to 3½ hr after intraperitoneal administration of various substances. All values are in micrograms per gram of fresh liver.

| Animal | Control† | Acetyl pyridine injected‡ | Nicotinamide injected§ |
|---|---|---|---|
| 1 | 450 | 1690 | 2840 |
| 2 | 408 | 1850 | 3420 |
| 3 | 482 | 1460 | 3140 |
| 4 | 350 | 1750 | 3050 |
| Average | 423 | 1690 | 3165 |

* Represents both DPN and TPN concentrations.
† Injected with 0.85 percent saline.
‡ Given 500 mg of acetyl pyridine per kilogram of body weight.
§ Given 500 mg of nicotinamide per kilogram of body weight.

As mentioned in a preceding paragraph, the acetyl pyridine analog of DPN was detected in both brain and spleen (*21*). The highest concentration of analog after the injection of acetyl pyridine was found in neoplastic tissues. Three different strains of tumors (Leukemia L1210, Bashford Carcinoma, and Sarcoma 37) all yielded relatively large amounts of the analog after administration of the antimetabolite. Table 4 gives representative data with the leukemia. Nicotinamide injection produced some increase in the DPN content of the leukemia. Acetyl pyridine administration, in contrast to the picture in liver, caused a decrease in the DPN level of the tumor. The decrease in DPN can be largely accounted for by synthesis of the analog (Table 4). When nicotinamide was given with the acetyl pyridine, a significant decrease in the amount of the analog occurred. Results similar to those in Table 4 were obtained with both Sarcoma 37 and Bashford Carcinoma.

The analog has been isolated from the leukemia by precipitation with acetone after removal of proteins with trichloroacetic acid. The DPN present was cleaved with Neurospora DPNase (*22*), which does not split the acetyl pyridine analog (*5*). The acetyl pyridine analog was identified by its reduced spectrum (maximum at 365 mμ) after reaction with yeast alcohol dehydrogenase and alcohol (*5*).

Whether the toxic effects of acetyl pyridine are manifested in the analog itself or are caused by a decrease in the DPN level resulting from formation of the analog is not yet clear. Attempts to clarify this point have been undertaken by injection of the 3-acetyl pyridine analog of DPN itself. Preliminary experiments indicate that the DPN analog is not as toxic as free acetyl pyridine. Work is now also in progress on the activities of acetyl pyridine and the acetyl pyridine analog in tumor therapy.

It is our view that the demonstration of the forma-

Table 4. Effect of acetyl pyridine and nicotinamide injection on the pyridine nucleotide content of mouse leukemia. Animals were given various intraperitoneal injections and were sacrificed 3 hr after injection. All values are in micrograms per gram of fresh tissue.

| Animal | Control* | Nicotinamide† | Acetyl pyridine‡ | | Acetyl pyridine and nicotinamide§ | |
|---|---|---|---|---|---|---|
| | DPN | DPN | DPN | Analog | DPN | Analog |
| 1 | 141 | 360 | 87 | 49 | 311 | 19 |
| 2 | 131 | 321 | 102 | 49 | 145 | 11 |
| 3 | 150 | 326 | 102 | 44 | 204 | 19 |
| 4 | 150 | 282 | 55 | 33 | | |
| 5 | 136 | 282 | 93 | 49 | | |
| Average | 141 | 322 | 86 | 42 | 220 | 17 |

* Injected with saline.
† Injected with 1000 mg nicotinamide per kilogram of body weight.
‡ Injected with 1000 mg acetyl pyridine per kilogram of body weight.
§ Injected with both 1000 mg nicotinamide and 1000 mg acetyl pyridine per kilogram of body weight.

tion of a coenzyme analog in the intact animal is of importance in elucidating a system of chemotherapy. Coenzymes are the functional forms of the vitamins and, as such, they occupy key positions in metabolism. A decrease in effective concentration by the formation of analogs would be expected to interfere with the normal functions of cells. This is illustrated by the metabolic disturbances that are associated with vitamin deficiency. Exchange reactions that involve coenzymes appear to be a rapid means of producing coenzyme analogs *in vivo* and, as a result, a rapid means for inhibiting or altering cellular metabolism. This is illustrated by the marked acute toxicity of such a compound as acetyl pyridine. An appreciation of the antimetabolite-metabolite relationship as applied to enzymes catalyzing exchange reactions is highly desirable, and it is to be hoped that further work on this problem not only will be of value from the viewpoint of chemotherapy but also will aid us to understand the significance of enzymatically catalyzed exchange reactions.

## Summary

1) 3-Acetyl pyridine has been found to be quite toxic when administered to mice. Simultaneous administration of nicotinamide or DPN protects against the toxicity of acetyl pyridine. Nicotinic acid and tryptophan do not protect animals from the lethal effects of the compound.

2) Nicotinamide inhibits the formation of the 3-acetyl pyridine analog of DPN from acetyl pyridine and DPN in mouse brain homogenates. Nicotinic acid has no inhibitory effect on analog synthesis.

3) Administration of acetyl pyridine results in a fourfold increase in the DPN level of the lever. No acetyl pyridine analog of DPN is found in the liver after injection of the antimetabolite.

4) Injection of acetyl pyridine into tumor mice results in the formation of the acetyl pyridine analog of DPN in the neoplastic tissues. A decrease in DPN concentration is associated with the analog synthesis. The DPN analog has been isolated from tumor tissue and identified.

5) The results are discussed with respect to the significance of enzymatically catalyzed exchange reactions in chemotherapy.

### References and Notes

1. The abbreviation DPN is used for diphosphopyridine nucleotide. In the formula NRPPRA, N is nicotinamide, R is ribose, P is phosphate, A is adenine, X is a pyridine compound related to nicotinamide.
2. L. J. Zatman *et al.*, *J. Biol. Chem.* **209**, 453 (1954).
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5. N. O. Kaplan and M. M. Ciotti, *ibid.* **76**, 1713 (1954).
6. ———, in preparation.
7. Contribution No. 93 of the McCollum-Pratt Institute. This work was supported in part by grants from the American Cancer Society as recommended by the Committee on Growth of the National Research Council, the Williams Waterman Fund, the American Trudeau Society Medical Section of the National Tuberculosis Association, and the Rockefeller Foundation.
8. D. W. Woolley, *J. Biol. Chem.* **157**, 455 (1945).
9. A. Goldin, S. R. Humphreys, and J. M. Venditti, in preparation.
10. It is of interest to note that Ackermann and Taylor (*11*), in their studies of the toxicity of acetyl pyridine on embryonic chicks, found that nicotinamide reversed the toxicity competitively, whereas nicotinic acid and tryptophan had only a slight effect. Furthermore, in Woolley's experiments (*8*), a protective action of nicotinic acid on acetyl pyridine toxicity in mice occurred only when the acid was prefed. Simultaneous administration of the acid with the acetyl pyridine was not effective.
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12. L. J. Zatman, N. O. Kaplan, and S. P. Colowick, *J. Biol. Chem.* **200**, 197 (1953).
13. $LD_{50}$'s and standard error calculated by Kärber's method as described by Cornfield and Mantel (*14*).
14. J. Cornfield and N. Mantel, *J. Am. Stat. Assoc.* **45**, 181 (1950).
15. N. Mantel, personal communication.
16. E. G. McDaniel, *Federation Proc.* **12**, 472 (1953).
17. O. H. Gaebler and W. T. Beher, *J. Biol. Chem.* **188**, 343 (1951).
18. W. T. Beher, W. M. Holliday, and O. H. Gaebler, *ibid.* **198**, 573 (1953).
19. W. T. Beher and W. L. Anthony, *ibid.* **203**, 895 (1953).
20. A detailed description of the conditions and kinetics that are involved in the conversion of nicotinamide to DPN will be given elsewhere.
21. The acetyl pyridine analog was determined by its resistance to Neurospora DPNase. The method used for this determination, as well as that for DPN, will be described in detail in a subsequent publication.
22. N. O. Kaplan, S. P. Colowick, and A. Nason, *J. Biol. Chem.* **191**, 473 (1951).