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INTERACTION OF NICOTINAMIDE WITH RESERPINE AND CHLORPROMAZINE

III. Some effects on the diphosphopyridine nucleotide content in liver (1), (2)

BY

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It has been observed by HOAGLAND (14) and BURCH (7) that the administration of nicotinic acid produces an increase in diphosphopyridine nucleotide (DPN) levels of blood. KAPLAN and coworkers have found that the injection of nicotinamide into mice results in large increases of the level of DPN in liver, brain and spleen (16). For example, in liver the DPN level may increase as much as 10 fold within a 12 hour period following the injection of 500 mg/kg of nicotinamide. Within 24 hours, however, the pyridine nucleotide level of the liver is found to have returned to its basal value. BURTON *et al.* (8) have shown that the administration of tranquillizing agents such as reserpine and chlor-

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promazine prior to the injection of nicotinamide results in the pyridine nucleotide content of liver being maintained at an elevated concentration for a prolonged time. This paper reports the details of these observations and shows that only those compounds possessing tranquillizing activity are effective in maintaining an elevated DPN level. Some of the possible implications of these observations are discussed.

Methods

Hybrid male mice (BALB *c* An × DBA 2J) F_1, 8 to 10 weeks old, 20 to 25 grams body weight, were divided in a random manner into groups of appropriate size for the experiments reported. A few experiments were performed using DBA male mice obtained from the Rockland Farm. In general, the tranquillizing drugs and other compounds administered were given subcutaneously 4 hours prior to the intraperitoneal injection of nicotinamide. Food and water were withheld from all animals following the administration of the drugs. At the indicated time interval following the injection of nicotinamide (500 mg/kg), the mice were killed by cervical fracture. The livers were rapidly removed, weighed, and homogenized in cold 5 % trichloroacetic acid (16). Pyridine nucleotide analyses were carried out employing the modified method of Levitas *et al.* (11, 16, 17).

Results

Effect of Reserpine and Related Compounds on DPN Content of Liver. The data presented in Table I show that as a result of the administration of reserpine or compounds related to reserpine that liver DPN levels 24 hours after the injection of nicotinamide were increased. The first line in the table (indicated by the heading, NONE) presents a number which represents the difference between the DPN levels of a control group of animals untreated in any way and of a group of animals 24 hours following the administration of only nicotinamide. The second group refers to those control animals which had received the vehicle used for the reserpine derivatives and nicotinamide. It is apparent that neither control groups differed significantly from the values of the reference animals, i.e., those receiving nicotinamide alone. Of all the compounds employed related to reserpine, only reserpine and deserpidine were effective in maintaining a high pyridine nucleotide level in liver. The

other drugs employed, such as rescinnamine, methyl reserpate, reserpic acid, and yohimbine were without effect. It should be noted that rescinnamine is a tranquillizing agent in some animals, but was without effect in producing sedation or tranquillization in the mouse. A stereoisomer of reserpine without tranquillizing effect, 3-isoreserpine, was unable to maintain high liver DPN levels when employed as the pure reserpine-free isomer. The value reported in Table I refers to a preparation of 3-isoreserpine that has since been found to contain 5 % reserpine. It should be further noted that the drugs employed in this study were administered at equimolar doses. The inactive preparations have been given at doses up to those levels which produce death and are without effect on the liver DPN levels. MILLER and WEINBERG (18) reported that the diethylaminopropanol ester of trimethoxybenzoic acid is an effective tranquillizer (1) and may, therefore, be the functional part of the reserpine molecule. PALAZZO and coworkers (19) recently studied a series of derivatives of trimethoxybenzoic acid for tranquillizing abilities. One of these compounds, β-morpholine-α-methyl ethanol ester of 3, 4, 5 trimethoxybenzoic acid (Compound XV), was observed in their animal assay to be a tranquillizing agent (2) (19). This compound was studied in our system (using CDBA mice) in doses from 50 to 200 mg/kg (approximately the LD_{50}) without any effect on liver concentration of DPN. Another strain (DBA mice; Rockland Farms) did respond to compound XV at 200 and 250 mg/kg, producing elevation in liver DPN comparable to that produced by reserpine. Sodium trimethoxybenzoate was unable to maintain high liver DPN levels, and was without any sedative action.

The effect of phenothiazine derivatives on DPN content of liver. The data presented in Table II show the effect of administering various phenothiazine derivatives to mice prior to the administration of nicotinamide. The values are the increase in liver DPN levels measured at 24 hours over that of the liver DPN levels of mice receiving nicotinamide alone. The value representing untreated control mice is found to show no significant difference from those mice receiving nicotinamide alone. Chlorpromazine, acetyl promazine, promazine, WY-1160, WY-1114, WY-1121, WY-1137, prochlorperazine, and trifluoperazine produced an increase in liver DPN levels. All of these derivatives are effective in tranquillizing morphine mania of cats except WY-1114(2*a*). All, including

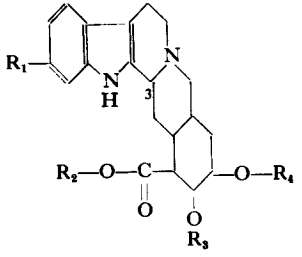
(1) Indicated by ability to prolong barbiturate anesthesia.

(2) Indicated by ability to (1) reduce spontaneous activity, (2) reduce activity due to amphetamine, and (3) prolong barbiturate anesthesia.

TABLE I

Effect of Reserpine and Related Compounds on DPN Content in Liver. The experiment was performed essentially as described in the methods section. Reserpine (10 mg/kg; 16 μmol/kg) and all the other compounds (equimolar dose, i.e. 16 μmol/kg) were administered intraperitoneally 4 hours prior to the intraperitoneal injection of nicotinamide (500 mg/kg). Animals were killed at 12, 24 and 36 hour intervals following nicotinamide, the livers rapidly removed, and the DPN content determined. In all cases, the 12 hour DPN levels were high (data not shown, cf. TABLE V). The data reported are for the 24 hour interval and represent the increase in DPN level over that level in animals receiving only nicotinamide. The values reported are the average values obtained from at least three mice

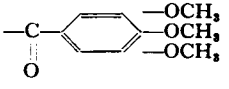
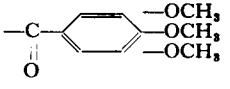
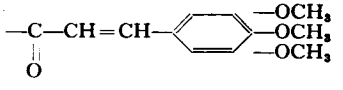
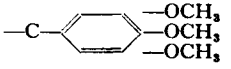
Effect of Reserpine and Related Compounds on Diphosphopyridine Nucleotide Synthesis



Compound	Group Substitution				Increase in Liver Diphosphopyridine Nucleotide
	R_1	R_2	R_3	R_4	μg/g Wet Weight
None					30
Vehicle**					20

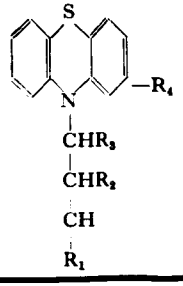
** Vehicle For All the Heterocyclic Bases : 2.4 % Glacial Acetic Acid, 2.4 % Ethanol, 12.2 % Propylene Glycol, and 83.0 % Water.

TABLE I *(continued)*

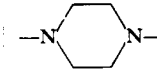
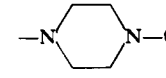
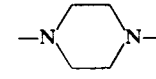
Compound	Group Substitution				Increase in Liver Diphosphopyridine Nucleotide
	R_1	R_2	R_3	R_4	μg/g Wet Weight
Reserpine	—OCH_3	—CH_3	—CH_3		1550
Deserpidine	—H	—CH_3	—CH_3		2220
Rescinnamine	—OCH_3	—CH_3	—CH_3		—20
3-Isoreserpine***	—OCH_3	—CH_3	—CH_3		430
Methyl Reserpate	—OCH_3	—CH_3	—CH_3	—H	—70
Reserpic Acid	—OCH_3	H	—CH_3	—H	—50
Yohimbine	—H	—CH_3	—H	—H (Replacing —OR_4)	90
3-epi-α-Yohimbine	—H	—CH_3	—H	—H (Replacing —OR_4)	40

*** This Preparation Consisted of 95 % 3-Isoreserpine and 5 % Reserpine.

TABLE II



Effect of Phenothiazine Derivatives on DPN Content in Liver. The experimental details are described in methods and table I. Chlorpromazine (10 mg/kg) and the other compounds were given in equimolar doses (28 μmols/kg) four hours prior to the injection of nicotinamide. The data reported refer to the increment between values from experimental animals and control values at 24 hours following the nicotinamide. All phenothiazine derivatives were administered in aqueous solutions. Experiment I was performed with CxDBA male mice, while DBA mice were used in Experiment II

Compound	Group Substitution				Increase in Liver Diphosphopyridine Nucleotide
Experiment I	R_1	R_2	R_3	R_4	μg per g wet weight
None					65
Chlorpromazine	$-N(CH_3)_2$	—H	—H	—Cl	1710
Acetylpromazine	$-N(CH_3)_2$	—H	—H	$-C(=O)-CH_3$	1370
Promazine	$-N(CH_3)_2$	—H	—H	—H	1580
WY-1160	$-N(CH_3)_2$	—H	$-CH_3$	—H	1340
Phenergan	—H	$-N(CH_3)_2$	—H	—H	330
WY-1114	$-N(CH_2CH_2CH_3)_2$	—H	—H	—H	1400
WY-1121	—N	—H	—H	—H	1620
WY-1137	—N	—H	—H	—H	1800
Experiment II					
None					85
Chlorpromazine	$-N(CH_3)_2$	—H	—H	—Cl	749
Prochlorperazine		—H	—H	—Cl	315
Trifluoperazine (SKF 5019)		—H	—H	$-CF_3$	402
Thioperazine (SKF 5883)		—H	—H	$-SO_2N(CH_3)_2$	1
Phenothiazine	(No substitution on Ring Nitrogen)			—H	34

WY-1114, produced sedation in mice. It is interesting to note that phenergan, which is a less effective tranquillizer than chlorpromazine, is also less effective (on an equimolar basis) in maintaining elevated DPN levels: chlorpromazine produced a 1700 μg/g increase in liver DPN, whereas phenergan produced a 330 μg/g increase. Methoxypromazine (not included in TABLE II) at high dose levels (100-500 mg/kg) was effective in maintaining elevated DPN levels. Interestingly enough, trifluoperazine, which is an effective tranquillizing agent in humans at dosages 1/50th to 1/100th of chlorpromazine, was no more effective in our test system than chlorpromazine. At low dose levels trifluoperazine showed no effect upon nicotinamide-induced high DPN levels and failed to produce tranquillization of the mice or rats employed. At dose levels comparable to chlorpromazine (50 to 200 mg/kg) trifluoperazine was found to produce tranquillization of the animals and to maintain elevated liver DPN levels. The sulfonamide derivative, thioperazine, is a phenothiazine antiemetic without tranquillizing ability and inactive in our assay system. The parent compound, phenothiazine, was not able to maintain the elevated DPN levels, nor does it have a sedative action.

The effect of a variety of compounds on DPN content in liver. Table III presents data which show the effect of a number of chemically unrelated

TABLE III

The Effect of a Variety of Compounds on Diphosphopyridine Nucleotide Content in Liver

Experimental details are given in Methods, Tables I and II and the text

Compound	Dose	Diphosphopyridine Nucleotide
	mg/kg	μg/g wet weight liver
Control (Nicotinamide only)	—	410
Reserpine	10	2220
Chlorpromazine	200	1250
Meprobamate	400	410
Phenobarbital	200	560
Ethanol	2000	330
Benactyzine	10	360
Sempervirine Nitrate	10	320
Serpentine Nitrate	10	420
Ajmaline	10	320
Echitamine HCl	10	380
Afraserpine	10	440

compounds on the nicotinamide-elevated DPN content in mouse liver. It can be seen that the only two drugs listed which maintained the elevated DPN level were reserpine and chlorpromazine. Other drugs, which produced sedation at the levels used, such as phenobarbital, ethanol and meprobamate, failed to maintain elevated DPN levels. These drugs were administered in divided doses to maintain a state of inactivity comparable to the reserpine and chlorpromazine treated animals. Other drugs such as benactyzine (a " psychic energizer "), chlormethazanone (a centrally acting skeletal muscle relaxant), and a number of other chemically unrelated compounds were without effect on DPN levels.

Chlorpromazine dose-response relationship. The relationship between the dose of chlorpromazine administered and the nicotinamide-elevated levels of DPN in liver is illustrated by the data in Table IV. Chlorpro-

TABLE IV

Dose-Response Relationship for the Effect of Chlorpromazine on Maintaining Nicotinamide-elevated Diphosphopyridine Nucleotide Content in Liver

The experimental details are described in Methods, Table I and II, and the text

Chlorpromazine	Diphosphopyridine Nucleotide	
	24 hours	36 hours
mg/kg	μg/g wet weight liver	
0	350	310
19	1020	310
32	1800	350
54	1840	460
90	1900	480
125	1700	520
250	1940	1200

mazine at a dose of 19 mg/kg gave a half maximal increase 24 hours following the nicotinamide administration. Maximal elevation occurred between 30 and 50 mg/kg. The only effect of increasing the dose of the tranquillizing agent is to produce a more prolonged effect rather than a greater increase in the DPN level of the liver. Thus, 36 hours after the administration of nicotinamide, 30 mg/kg of chlorpromazine failed to maintain the elevated DPN levels, while some increase may be observed at chlorpromazine dosages between 50 and 125 mg/kg. At 250 mg/kg, the 36 hour level is considerably elevated being 1200 μg/g compared to the maximal level of 1700 to 1900 μg/g wet weight liver.

The dose response relationship for reserpine has been reported previously (8).

The effect of sequence and the time of administration of reserpine and nicotinamide on DPN levels. Table V presents data which illustrate the effect of reserpine administered before and after nicotinamide on the DPN levels of mouse liver. Neither the presence of reserpine nor the time sequence, i.e. 4 hours before or 4 hours after administering nicotinamide had any effect on the 12 hour DPN levels. (They were elevated in all three cases.). The administration of nicotinamide 4 hours

TABLE V

The Effect of Reserpine Administered Before and After Nicotinamide on Diphosphopyridine Nucleotide Content in Liver

The experimental details are described in Methods, Tables I and II, and the text

	Diphosphopyridine Nucleotide		
	Hours after Nicotinamide		
	12	24	36
	μg per g wet weight liver (1)		
1. Nicotinamide (500 mg/kg)	1580	330	340
2. " 4 hours after Reserpine (10 mg/kg)	1980	2050	810
3. " 4 hours before Reserpine (10 mg/kg)	1600	670	350

(1) Each figure represents the average value of three mice.

after the reserpine resulted in the 24 hour DPN content of liver being as high as the 12 hour DPN level, while the 36 hour DPN level was still twice the basal level. The administration of the reserpine ¼ hours after the nicotinamide was almost without effect on the DPN content; 24 hours following the nicotinamide injection the liver DPN levels were 670 μg/g compared to the basal level of about 330-340, an elevation of only two-fold. While 36 hours after the nicotinamide injection, the DPN level had returned to the basal value. It is, therefore, apparent that the reserpine must be given before the nicotinamide, rather than after. The data presented graphically in Figure 1 show the effect of increasing the time interval between the reserpine and the subsequent nicotinamide injections on the DPN level of mouse liver 24 hours after the nicotinamide. Maximal effect was observed 4 hours after the reserpine injection

and decreased progressively for 36 hours until reserpine was no longer capable of maintaining the elevated DPN levels of liver. These data thus parallel the known delay in producing tranquillization by reserpine and in the long duration of effect of reserpine.

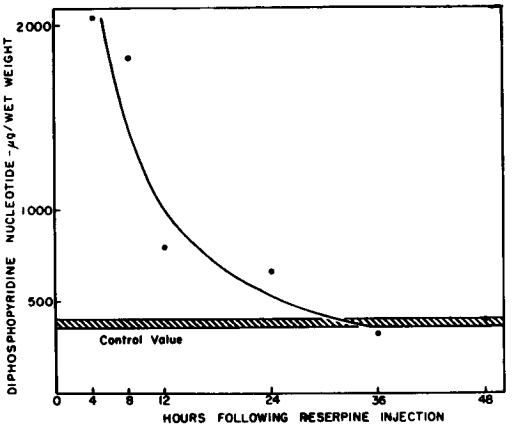


FIG. 1

The Effect of Increasing the Time Interval between the Administration of Reserpine and Nicotinamide. The experimental details are described in Methods, Table I, and the text. Reserpine (10 mg/kg) was administered at increasing time intervals prior to the Nicotinamide (500 mg/kg). The data presented represents the liver DPN levels 24 hours after the Nicotinamide was given. Each point represents an average of the values obtained from at least three mice.

TABLE VI

Effect of Lysergic Acid Diethylamide on Reserpine-Maintained Elevated DPN Levels

The experimental details are described in Methods, Tables I and II, and the text. LSD-25 (dose as indicated) was given intraperitoneally 15 minutes prior to the reserpine (5 mg/kg) injection. Nicotinamide (500 mg/kg) was administered 4 hours after the reserpine. The data reported are for the 24 hour DPN levels

Lysergic Acid Diethylamide	Diphosphopyridine Nucleotide	
	Control Reserpine Omitted	Reserpine Administered
mg per kg	μg per g wet weight liver	
0	360; 410	2180
0.6	570	1670
1.3	420	1740
2.5	390	2070
5.0	390	1860
10.	420	1910
20.	420	1670

Lysergic acid diethylamide. Lysergic acid diethylamide (LSD-25) has been reported by a number of investigators to antagonize certain effects of reserpine and 5-hydroxytryptamine, such as the ability of reserpine to potentiate barbiturate sleep time and the ability of 5-hydroxytryptamine to stimulate uterine muscle contraction and contraction of carotid rings (12, 13, 20, 21, 22). The LSD-25-reserpine-5-hydroxytryptamine relationship has lead to speculation that reserpine action may be mediated via 5-hydroxytryptamine (5). It was of interest, therefore, to determine if LSD-25 had any effect upon the ability of reserpine to maintain nicotinamide-induced high DPN levels. The data presented in Table VI show that LSD had no effect on this property of reserpine, nor did LSD itself have any effect on the DPN levels of mouse liver.

Discussion

Some of the pharmacological effects of administering massive doses of nicotinamide have been reported by Bergmann and Wislicki (3) and includes a hypotensive action and hyperglycemia. A blood half-time of approximately 100-125 minutes was determined for nicotinamide (3). Recently, several central nervous system effects of massive doses of nicotinamide have been described. Such effects include sedation or tranquillization and the prolongation of barbiturate anesthesia (9, 10, 15, 23). From the biochemical point of view, Kaplan and his collaborators have observed that the injection of massive doses of nicotinamide into mice results in large increases of the level of DPN in tissues such as liver, brain and spleen (16). In these experiments, the DPN content increased 8 to 10-fold in liver within 12 hours, but by 24 hours the DPN level had returned to its normal basal value. The experiments reported in this paper show that there is a correlation of the ability of tranquillizing agents to act in the animal to maintain the nicotinamide-elevated DPN content of liver and their ability to tranquillize ([1]). Reserpine and related tranquillizing agents, as well as chlorpromazine and derivatives of phenothiazine which have tranquillizing ability, produced this effect; non-tranquillizing derivatives of reserpine and chlorpromazine were

([1]) In respect to tranquillization mechanisms, it would be of greater interest had such a correlation been shown in a study of brain tissue DPN levels. However, the DPN content of brain tissue is elevated to a much smaller extent than that of liver following nicotinamide administration; liver often shows an increase in its DPN content of 800 to 1000 %, while brain DPN levels may be elevated only 30 to 50 % (16). It has not been possible to demonstrate a consistent effect of reserpine and chlorpromazine on maintaining nicotinamide-elevated DPN content of brain tissue (8*a*).

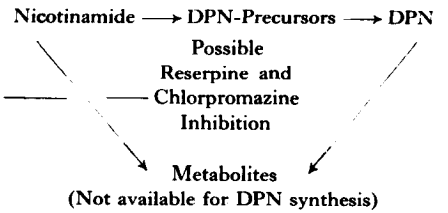
ineffective in maintaining the elevated DPN levels. Sedatives, such as phenobarbital, meprobamate, and ethanol, produce a state of inactivity comparable to that produced by reserpine and chlorpromazine derivatives, yet the sedatives fail to maintain nicotinamide-elevated DPN levels.

The data reported suggests the following structure-activity relationships for reserpine derivatives in respect to maintenance of elevated DPN levels: the 11-methoxy group is not essential to activity, the 18-hydroxy group must be esterified (trimethoxy-benzoyl ester is effective whereas the electronically similar cinnamyl ester is without activity in the mouse), the steric arrangement of carbon 3 is critical (reserpine is active, 3-isoreserpine has no activity). The 3, 4, 5-trimethoxy benzoic acid ester of β-morpholine-α-methyl ethanol was without activity in one strain of mice (CDBA), but showed activity in DBA mice. Sodium trimethoxybenzoate was unable to maintain the elevated DPN levels. Insofar as the phenothiazine derivatives are concerned, substitution at the 2 position of the phenothiazine nucleus is not essential for the maintenance of high DPN levels. Substitution on the phenothiazine nitrogen is essential; phenothiazine, itself, and methylene blue are inactive in this system. The position of the tertiary nitrogen on the *n*-propyl side chain is of some importance; in the 3′-position, as in promazine, the activity is high, while in the 2′-position, as in phenergan, this activity is considerably reduced. The nature of the substituents on the 3′ nitrogen appears to be non-critical.

The dose of reserpine necessary to produce half-maximal response is between 0.6 and 1.6 mg/kg, while that of chlorpromazine is approximately 19 mg/kg. Increasing the dose of reserpine or chlorpromazine above that necessary to produce maximal maintenance of the DPN level at 24 hours prolongs the time interval during which the DPN levels are elevated.

The biochemical basis for the effect of reserpine and chlorpromazine on the nicotinamide-produced elevation of the DPN content of tissue will now be considered. When nicotinamide is administered to animals, it may proceed through the following sequence, as outlined in scheme 1, i.e., nicotinamide yields precursors of DPN; these precursors can produce DPN, and DPN in turn can be degraded to free nicotinamide or metabolites of nicotinamide such as N^1-methyl nicotinamide, N^1-methyl nicotinic acid (trigonellin), various pyridones, etc. It has been found that after the injection into mice of large doses of nicotinamide, free nicotinamide is excreted by the animals (4). However, at a later time period (when the DPN concentration in the liver begins to decrease),

SCHEME 1



other excretory products appear in urine, whereas only traces of free nicotinamide can be detected. The possibility exists that metabolites of nicotinamide may arise directly from the cleavage of DPN. Following the injection of nicotinamide, high levels of DPN are produced because the rate of synthesis has exceeded the rate at which the enzymes degrade DPN. Reserpine and chlorpromazine could act by increasing the rate and/or duration of synthesis of DPN or by interfering with the degradation of DPN. It has been found that reserpine and chlorpromazine do not appreciably affect the rate of synthesis of DPN. The only effect, if any, is a slight inhibition of the rate of rise of the DPN content of liver (8). It is of significance that reserpine must be given before the nicotinamide for it to be effective in maintaining the elevated coenzyme levels (TABLE V). When nicotinamide is administered to mice, the DPN levels continued to increase for 8 to 10 hours. Reserpine administered 4 hours after nicotinamide, while the DPN levels are increasing, has little or no ability to keep the levels of DPN high. This observation can be interpreted to mean that reserpine does not affect the enzyme systems which degrade DPN. In addition, experiments conducted both *in vivo* and *in vitro* by LANGAN and by BONAVITA [1] show that reserpine and chlorpromazine do not act by interfering with the cleavage of DPN. While reserpine and chlorpromazine do not increase the rate of synthesis of DPN nor inhibit the pathways by which DPN is degraded, they may produce a prolonged synthesis of DPN by maintaining a supply of suitable DPN precursors or of nicotinamide. This could be accomplished by interference with enzyme systems that metabolize nicotinamide or other DPN precursors. Preliminary evidence suggests that chlorpromazine does increase the concentration of nicotinamide and DPN precursors at the 12 and 24 hour time periods. In addition, some observations have been made indicating that chlorpromazine is an inhibitor of nicotinamide methylpherase [2]. In this connection, it is of

[1] Dr. T. LANGAN and Dr. V. BONAVITA, Brandeis University, personal communication.
[2] Dr. R. A. SALVADOR, unpublished observations.

interest to note that AXELROD and his co-workers have shown that tranquillizing agents can interfere in the methylation of histamine (6).

In view of the correlation between the ability of drugs to tranquillize and their ability to maintain elevated DPN levels and the fact that non-tranquillizing sedatives such as phenobarbital and ethanol are ineffective in this biochemical system, it is tempting to speculate that the DPN levels themselves or the biochemical system affected which gives rise to the elevated levels may be involved in the mechanism of tranquillization. The possibility must be considered that the effect of chlorpromazine and reserpine on DPN levels in tissue may be only a reflection of a primary event in the mechanism of tranquillization rather than an integral part of the mechanism itself. For example, these drugs inhibit methylation enzyme systems which may lead to elevated DPN levels (as discussed above) and possibly to changes in levels of a neurohormone such as nor-epinephrine, which is inactivated by methylation (1, 2).

This work should aid both in the elucidation of the mechanism by which tranquillizing agents can maintain elevated DPN levels and should help to eluciate the pathways of biosynthesis of DPN in tissues such as liver (1). It should be emphasized, however, that the effect of the tranquillizing agents on brain may be different from the liver, since some of the reactions involving DPN and nicotinamide metabolism have, as yet, not been detected in the brain preparations.

SUMMARY

Massive doses of nicotinamide increase the DPN level of mice tissues. These elevated DPN levels quickly return to their basal value unless the animals have been treated with a chemical agent such as reserpine or chlorpromazine. A correlation has been obtained between the ability of reserpine and reserpine derivatives and chlorpromazine and phenothiazine derivatives to tranquillize animals and their ability to maintain elevated DPN levels. Non-tranquillizing sedatives such as phenobarbital and ethanol are not able to maintain these high DPN levels. Evidence has been presented which indicates that this action of the tranquillizing agents is not mediated through serotonin. Since reserpine and chlorpromazine do not affect the rate of DPN synthesis from nicotinamide and do not inhibit the degradation of DPN, it has been suggested that they interfere with the formation of metabolites of nicotinamide.

(1) While the pathway of DPN synthesis in erythrocytes has been thoroughly elucidated (PREISS, J. and HANDLER, P. *J. Biol. Chem.*, 1958, *233*, 488), the mechanism of DPN formation in liver and brain has not yet been fully clarified.

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