13. Tata, J. R., Brit. Med. Bull. **16**, 142 (1960).
14. Huang, K. C. and Knoefel, P. K., J. Pharmacol. Exptl. Therap. **121**, 443 (1957).
15. Nepomuceno, C. G. and Little, J. M., J. Pharmacol. Exptl. Therap. **146**, 294 (1964).
16. Goodman, L. S. and Gilman, A., "The Pharmacological Basis of Therapeutics." Macmillan, New York (1965).

Received Feb. 4, 1969. P.S.E.B.M., 1969, Vol. 131.

1-Methyl-1-nitrosourea Depression of Brain Nicotinamide Adenine Dinucleotide in the Production of Neurologic Toxicity (33914)

PHILIP S. SCHEIN[1] (Introduced by David P. Rall)
Laboratory of Chemical Pharmacology, National Cancer Institute, Bethesda, Maryland 20014

1-Methyl-1-nitrosourea (MNU) has been shown to be both a potent carcinogenic and antitumor compound (1, 2). These effects have been attributed to the liberation of diazomethane, a highly reactive agent capable of alkylating protein, RNA, DNA, and inhibiting the incorporation of amino acids into protein (3, 4). Recent investigations demonstrated that MNU can produce a rapid dose-related depression in liver nicotinamide-adenine dinucleotide (NAD) concentrations (5, 6). During the course of these studies there appeared a transient neurologic syndrome which was temporally related to the acute lowering of brain NAD levels. This communication correlates these findings with measurements of drug concentration in the acid-soluble fraction of brain, and with brain NAD glycohydrolase activity and histology.

Methods. Male albino mice, Swiss strain, weighing 20–25 g were used for all studies, and were maintained on Purina laboratory chow pellets and water *ad libitum*. 1-Methyl-1-nitrosourea, NCS-23909, and nicotinamide (Calbiochem) were prepared in distilled water. Streptozotocin, NSC-85998, a diabetogenic compound composed of the union of glucosamine and MNU at the carbon 2 position of the glucose moiety (7), was dissolved in 0.005 *M* citrate buffer, pH 4.0. The drugs were administered at a volume of 1 ml/100 g of body weight intravenously via the tail vein, or intraperitoneally. The NAD content of brain was assayed enzymatically using alcohol dehydrogenase (Sigma) after the organ was homogenized 1:5 weight:volume in 0.6 *N* perchloric acid at 4°, and the supernate was neutralized using 3 *N* KOH (8). The NAD glycohydrolase activity of brain homogenate was assayed using the method of Kaplan as modified by Waravdekar (9). The concentration of MNU in the acid-soluble fraction of brain, liver, and serum was measured colorimetrically using a modification of the Forist method for streptozotocin (10, 6). For histologic study of brain, mice were anesthetized with ethyl ether, and 10% formalin was perfused through the heart. Sections of brain and spinal cord were rapidly removed and fixed in formalin and embedded in paraffin. The cresyl violet, Weil-Weigert, and Klüver–Barrera stains were used for the study of Nissl granules, nerve cell morphology, and myelin sheaths (11). For comparison, the brains of four normal mice were prepared in the same manner.

Results. Within 2–5 hr after the intravenous injection of MNU, 100 mg/kg, 60% of the mice demonstrated episodes of tonic seizure activity characterized by a straightening of the spine and stiffening of the tail, while the fore and hind limbs were thrust posteriorly (Fig. 1). Each attack had a duration of 10–20 sec following which the animal would remain refractory to further seizure activity for a period of 5–15 min. The syndrome appeared spontaneously or could be elicited by introducing tactile or auditory stimulation. By 5 hr after the single adminis-

[1] Present address: Department of Medicine, Beth Israel Hospital, 330 Brookline Avenue, Boston, Massachusetts 02215.

FIG. 1. The characteristic posture assumed by mice undergoing a 1-methyl-1-nitrosourea-induced tonic seizure.

tration of the drug the mice no longer showed seizure activity. The animals remained neurologically normal until death from bone marrow depression, which occurred 4–5 days posttreatment. A second group of animals received intraperitoneal nicotinamide, 500 mg/kg, 5 min prior to intravenous MNU, 100 mg/kg. These nicotinamide-pretreated mice did not show any evidence of seizure activity, and in contrast appeared sedated, lying quietly on the cage bedding with little spontaneous activity.

After the injection of MNU, 100 mg/kg, there occured a 25% reduction in brain NAD concentration, decreasing from average control values of 215 to 167 μg/g by 2 hr (Fig. 2). This was followed by a gradual rise in coenzyme concentration with stabilization at approximately 180 μg/g for the next 48 hr. A single intraperitoneal dose of nicotinamide prior to intravenous MNU produced NAD levels significantly greater than control at the 1–6 hr time periods, with protection against NAD depression for at least 12 hr. By 24 and 48 hr the combination treatment group demonstrated a gradual fall in NAD concentration, approaching that observed in animals receiving MNU alone, while exhibiting no overt signs of neurologic toxicity.

An MNU concentration of 33 μg/g was recorded in the acid-soluble fraction of brain 15 min after the intravenous injection of 100 mg/kg (Table I). This compared with a serum concentration of 1550 μg/ml and a liver concentration of 30 μg/g for the same time period. The drug remained measurable in brain for 1 hr but could not be detected at 90 min. Streptozotocin administered intravenously at equal molar doses produced no neurologic toxicity and was not detected in brain.

There was no significant change in NAD glycohydrolase activity over the time periods corresponding to the fall in NAD concentration (Table II). In the group of mice receiving MNU alone there was a decrease in enzymatic activity from a mean control value of 0.86 to 0.58 units/mg of protein/hr at the 48-hr time period. There was no evidence that nicotinamide in any way inhibited brain NAD glycohydrolase activity.

Sections of all major levels of brain and spinal cord were examined at 4 hr, and 1, 4, and 8 days posttreatment for evidence of gross and microscopic pathology. There was no evidence of neuronal chromatolysis, necrosis, or inflamatory cell reaction in any of the specimens. This failure to find pathology cor-

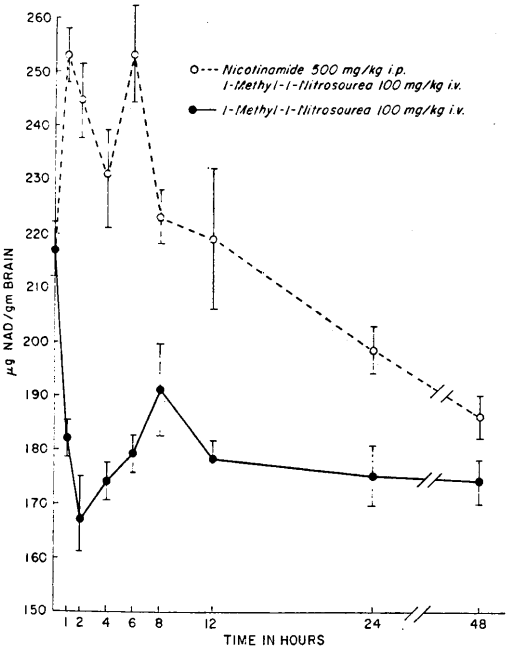

FIG. 2. Mouse brain NAD concentrations after the single injection of 1-methyl-1-nitrosourea, and the combination of nicotinamide 10 min prior to 1-methyl-1-nitrosourea. Each point represents the mean of 5 determinations ± 1 SD.

TABLE I. The Concentrations of 1-Methyl-1-nitrosourea in Mouse Brain after the Intravenous Injection of 100 mg/kg.

Time period (min)	Mean conc (μg/g of brain)[a]	± SD[b]
15	33	2
30	9	3
60	4	2
90	<1	

[a] Five determinations for all time periods.
[b] One standard deviation.

related with the complete recovery from neurologic toxicity.

Discussion. The pyridine nucleotides are known to be involved in many of the enzymatic reactions essential to cellular metabolism. With specific reference to the central nervous system, some attention has been directed to the role of NAD in the synthesis of acetylcholine (12) and the metabolism of gamma-aminobutyric acid (13). The neuropharmacologic interactions of these coenzymes have in large part been derived from the study of the action of antimetabolites of nicotinic acid and nicotinamide.

3-Acetylpyridine and 6-aminonicotinamide are capable of substituting for the usual vitamine constitutent in pyridine nucleotides, the exchange being carried out by the enzyme NAD glycohydrolase. The result is the formation of fraudulent analogs of NAD and NADP with subsequent inhibition of certain NAD-dependent enzyme systems (14). The principal drug effect noted in rodents, dogs, and monkeys has taken the form of neurologic toxicity (15–17). 6-Aminonicotinamide has been given a limited clinical trial in man as a cancer chemotherapeutic agent (18, 19). At moderate doses patients developed irreversible eighth nerve deafness, while at higher dose levels disorientation and ataxia appeared associated with electroencephalographic abnormalities.

MNU, though not a structural analog of nicotinamide, may serve as a experimental tool for the neuropharmacologic study of pyridine nucleotides. In the present study MNU has been shown to be capable of depressing brain NAD concentrations, with the onset of a tonic-clonic seizure disorder temporally related to the nadir of the coenzyme concentration. It is likely that the drug reduces brain NAD level by inhibition of *de novo* synthesis of pyridine nucleotides, since the principal degradative pathway, NAD glycohydrolase, was not activated. The mechanisms proposed for the elevation of NAD produced by nicotinamide include the direct incorporation of the vitamin through synthetic pathways (20), and its role as a noncompetitive inhibitor of NAD glycohydrolase (21). There was no evidence to support the latter phenomenon in this study, at the dose level of nicotinamide employed.

Mice which were pretreated with nicotinamide were protected against both the decrease of NAD and the overt neurologic toxicity. While nicotinamide might be serving as a nonspecific depressant of central nervous system activity, it is possible that there is a direct relationship between these clinical and chemical phenomena. This is suggested by the fact that the neurologic toxicity occured at a time when the drug was no longer present in detectable concentrations, and is

TABLE II. The NAD Glycohydrolase Activity in Mouse Brain after the Injection of 1-Methyl-1-nitrosourea, and the Combination of Nicotinamide 10 min Prior to 1-Methyl-1-nitrosourea.

Treatment	Time period (hr)	NAD glycohydrolase[ab] (units/mg of protein)	± SD[c]
Control	—	0.86	0.07
1-Methyl-1-nitrosourea 100 mg/kg, iv	1	0.76	0.05
	2	0.74	0.13
	4	0.86	0.25
	24	0.70	0.07
	48	0.58	0.06
Nicotinamide 500 mg /kg, ip + 1-Methyl-1-nitrosourea 100 mg/kg, iv	1	0.82	0.04
	2	0.87	0.26
	4	0.90	0.14
	24	0.74	0.08
	48	0.73	0.09

[a] Five determinations for each time period.
[b] One unit of enzyme is defined as that amount which will catalyze the transformation of one micromole of NAD per hour.
[c] One standard deviation.

further supported by the absence of correlative histopathology. Streptozotocin, composed of the union of glucosamine and MNU at the carbon 2 position, was not detected in brain and failed to produce either NAD depression or overt neurologic toxicity. It appears likely that the glucose carrier modified the inherent lipoid solubility of the MNU molecule and prevented passage through the blood–brain barrier (2).

1. Druckrey, H., Ivankovic, S., and Preussmann, R., Z. Krebsforsch. **66**, 389 (1965).

2. Schabel, F. M., Jr., Johnston, T. P., McCaleb, G. S., Montgomery, J. A., Laster, W. R., and Skipper, H. E., Cancer Res. **23**, 725 (1963).

3. Magee, P. N. and Farber, E., Biochem. J. **83**, 114 (1962).

4. Brouwers, J. A. and Emmelot, P., Exptl. Cell. Res. **19**, 467 (1960).

5. Schein, P. S. and Loftus, S., Cancer Res. **28**, 1501 (1968).

6. Schein, P. S., Cancer Res., in press. June, 1969.

7. Herr, R. R., Jahnke, H. K., and Argoudelis, A. D., J. Am. Chem. Soc. **89**, 4804 (1967).

8. Klingenberg, M., *in* "Methods of Enzymatic Analysis" (H. V. Bergomeyer, ed.,), p. 528. Academic Press, New York (1963).

9. Waravdekar, V. S. and Griffin, C. C., Exptl. Cell Res. **33**, 450 (1964).

10. Forist, A. A., Anal. Chem. **36**, 1338 (1964).

11. Klüver, H. and Barrera, E., J. Neuropathol. Exptl. Neurol. **12**, 400 (1953).

12. Harpur, R. P. and Quastel, J. H., Nature **164**, 779 (1949).

13. Burton, R. M., *in* "Inhibition in the Nervous System and Gamma-Aminobutyric Acid," p. 249. Macmillan (Pergamon), New York (1960).

14. Dietrich, L. S., Friedland, I. M., and Kaplan, L. A., J. Biol. Chem. **233**, 964 (1958).

15. Coggeshall, R. E. and MacLean, P. D., Proc. Soc. Exptl. Biol. Med. **98**, 687 (1958).

16. Sternberg, S. S. and Phillips, F. S., Bull. N. Y. Acad. Med. **35**, 811 (1959).

17. Wolf, A. and Cowen, D., Bull. N. Y. Acad. Med. **35**, 814 (1959).

18. Herter, F. P., Weissman, S. G., Thompson, H. G., Jr., Hyman, G., and Martin, D. S., Cancer Res. **21**, (1961).

19. Perlia, C. P., Kofman, S., Sky-Peck, H., and Taylor, S. G., Cancer **14**, 644 (1961).

20. Preiss, J. and Handler, P., J. Biol. Chem. **233**, 488 (1958).

21. Zatman, L. J., Kaplan, N. O., and Colowick, S. P., J. Biol. Chem. **200**, 197 (1953).

Received Feb. 6, 1969. P.S.E.B.M., 1969, Vol. 131.