1948, v25, 131.

12. Solomon, F., Diabetes, 1959, v8, 45.

13. Kim, J. N., Runge, W., Wells, L. J., Lazarow, A., ibid., 1960, v9, 396.

14. Osler, M., Acta Endocrinol., 1960, v34, 277.

15. Fee, B. A., Weil, W. B., Jr., Ann. N. Y. Acad. Sci., 1963, v110, 869.

16. Baird, J. D., Farquhar, J. W., Lancet, 1962, v1, 71.

17. Milner, R. D. G., Hales, C. N., Brit. Med. J., 1965, v1, 284.

18. Lieberman, L. L., Byul. Eksperim. Biol. i Med., 1964, v58, 46.

19. Stimmler, L., Brazie, J. V., O'Brien, D., Lancet, 1964, v1, 137.

20. Hales, C. N., Randle, P. J., Biochem. J., 1963, v88, 137.

21. Morgan, C. R., Sorenson, R. L., Lazarow, A., Diabetes, 1964, v13, 579.

22. Arquilla, E. R., Alexander, J. D., ibid., 1962, v11, 412.

23. Nelson, N., J. Biol. Chem., 1947, v153, 375.

24. Maline, J. M., Fitzgerald, M. G., Diabetes, 1965, v14, 175.

25. Kyle, G. C., Ann. Int. Med., 1963, v59, suppl. 3, 62.

26. Davey, D. A., Joplin, G. F., Santander, R., Lancet, 1961, v2, 71.

27. Buse, M. G., Roberts, W. J., Buse, J., J. Clin. Invest., 1962, v41, 29.

28. Jackson, W. P. U., Diabetes, 1960, v9, 373.

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Stimulation of Detoxification of O-Ethyl O-(4-Nitrophenyl) Phenylphosphonothioate (EPN) by Nikethamide and Phenobarbital.* (30697)

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DiStefano *et al*(1) demonstrated that the acute toxicity of the insecticide, O-ethyl O-(4-nitrophenyl) phenylphosphonothioate (EPN), to rats was reduced to a much greater extent by a combination of nikethamide and atropine than by atropine alone. Rosenberg and Coon(2) found that nikethamide greatly reduces the anticholinesterase action of EPN *in vivo* but the exact mechanism by which this central nervous system stimulant produces its protective effect has remained obscure.

Neal and DuBois(3) recently found that the detoxification of EPN to yield p-nitrophenol involves the action of a microsomal oxidase system in the liver, and developed a quantitative method for measuring the activity of this system. Brazda and Baucum(4) have demonstrated that nikethamide induces synthesis of a microsomal enzyme that catalyzes the oxidation of pentobarbital. It thus seemed possible that the protective action of nikethamide against EPN poisoning might involve induced synthesis of the detoxification system for EPN. The results of the present study demonstrated that treatment of rats with nikethamide increases the ability of their livers to detoxify EPN *in vitro*. Phenobarbital, a known stimulant of microsomal enzyme induction(5), also increased activity of the detoxification system and reduced the acute toxicity of EPN to rats.

Materials and methods. Adult, male and female Sprague-Dawley rats were used. EPN was kindly supplied by the E. I. duPont Co., Wilmington, Del. For administration to animals, EPN and nikethamide were dissolved in 20% ethanol and 80% propylene glycol. Sodium phenobarbital was dissolved in water. All drugs were given by the intraperitoneal route and the concentrations of solutions were adjusted so that the animals received amounts equivalent to 0.1% of the body weight. The doses of nikethamide and phenobarbital used for enzyme induction were 100 mg/kg/day and 50 mg/kg/day respectively. The rate of degradation of EPN to p-nitrophenol was measured by the method of Neal and DuBois (3) using whole liver homogenates. LD_{50} values were calculated from the 14-day mor-

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TABLE I. Stimulant Action of Nikethamide and Phenobarbital on Detoxification of EPN by Livers of Adult, Female Rats.

Days of treatment	Total dose (mg/kg)	Enzyme activity (μg p-nitrophenol/50 mg liver/hr) Avg	Range
	Nikethamide		
Control	0	2.5	(2.1- 3.1)
1	100	4.3	(4.1- 5.1)
2	200	5.7	(4.6- 8.0)
3	300	6.0	(5.6- 8.2)
5	500	5.9	(4.3- 6.9)
7	700	6.8	(6.0- 8.2)
10	1,000	6.3	(4.8- 6.4)
	Phenobarbital		
Control	0	2.5	(2.1- 3.1)
1	50	4.0	(3.6- 4.9)
2	100	5.5	(5.3- 5.8)
3	150	6.8	(4.9- 9.5)
5	250	9.9	(8.1-14.9)
7	350	9.0	(8.2-10.3)
10	500	11.6	(10.0-13.3)

tality data by the method of Litchfield and Wilcoxon(6).

Results. Table I shows the effects of daily administration of nikethamide (100 mg/kg) and phenobarbital (50 mg/kg) for periods up to 10 days on the activity of the microsomal enzyme system that catalyzes the degradation of EPN to p-nitrophenol. The animals were always sacrificed 24 hours after the last dose of each drug. Each value in the Table is the average and range for groups of 4 adult, female rats. The data show that treatment with nikethamide caused an increase in the ability of the liver to detoxify EPN with the maximum increase to 2 to 3 times the normal activity being observed after 3 days of treatment. Phenobarbital also markedly increased the enzyme activity to 4 times the normal level after 5 days of treatment.

A similar increase in enzyme activity was observed in adult male rats. The normal activity for males was 6.4 (5.2-8.17) μg of p-nitrophenol/50 mg of liver/hr. Treatment of males with nikethamide daily caused an increase in activity to 13.7 (13.5-14.0) in 3 days and there was no further increase in 5 days of treatment. Phenobarbital treatment of male rats resulted in a progressive increase in enzyme activity over a 5-day period to 20.6 (17.4-23.9) μg of p-nitrophenol/50 mg liver/hr.

To measure the rate of increase and reversal of the change in activity of the EPN detoxification system, single doses of nikethamide (100 mg/kg) or phenobarbital (50 mg/kg) were given intraperitoneally to female rats. Groups each containing 4 animals were sacrificed at 6, 12, and 24 hours and at later intervals during a period of 7 days after treatment with the drugs for measurement of the activity of the EPN detoxification system. The increase in enzyme activity following a single dose of nikethamide was not evident at 12 hours but it reached a maximum in 24 hours and the activity returned to normal in 3 days. With a single dose of phenobarbital, induced enzyme activity was apparent at 12 hours and reached a maximum in 24 hours. Reversal of the effect was relatively slow after phenobarbital treatment since it required 7 days for the activity to return to normal. Animals treated for 3 days with nikethamide exhibited reversal of the increase in enzyme activity to the normal level at 5 days after the last dose whereas in animals treated with phenobarbital for 3 days the activity was still slightly elevated at 7 days after the last dose.

Following measurements of the stimulant action of nikethamide and phenobarbital on the enzymatic detoxification of EPN, the toxicity of EPN was measured under conditions of treatment with the two drugs which produced maximal increases in the detoxification system as determined by the enzyme assays described above. Thus male and female rats were treated with 100 mg/kg of nikethamide or 50 mg/kg of phenobarbital for 5 days. At 24 hours after the last dose of each drug, the LD_{50} of EPN was measured. The results of these measurements are summarized in Table II. Comparison of the LD_{50} values for the control and drug-treated animals or the ratios of these values indicates that nikethamide and phenobarbital decrease the susceptibility of rats to the acute toxicity of EPN. A relationship was observed between the effectiveness of each drug in stimulating an increase in activity of the detoxification system (Table I) and in decreasing susceptibility of the animals to EPN.

TABLE II. Effect of Treatment with Nikethamide and Phenobarbital on Toxicity of EPN.

Treatment	Sex	No. of rats	I.P. LD_{50} (mg/kg)	19/20 confidence limits (mg/kg)	Ratio of LD_{50} drug-treated/ controls
Control	♀	44	7.3	(6.2- 8.5)	
	♂	42	33.0	(26 - 43)	
Nikethamide	♀	48	21.0	(16.6- 26.5)	2.9
	♂	44	90.0	(75 -108)	2.7
Phenobarbital	♀	48	75.0	(63 - 89)	10.3
	♂	44	125.0	(109 -142)	3.8

Phenobarbital was the most effective stimulant of the enzyme system and it was also the most effective in reducing the toxicity of EPN. Since the toxicity of EPN was measured 24 hours after the last dose of phenobarbital when the mild pharmacological actions of 50 mg/kg of phenobarbital had subsided, it seemed unlikely that reduction of the toxicity of EPN (Table II) involved any direct action of phenobarbital. Support for this conclusion was obtained by measuring the LD_{50} of EPN to female rats treated 30 minutes earlier with a single dose of 50 mg/kg of phenobarbital. Under these conditions the pharmacological actions of phenobarbital were maximal for the dosage used and the latent period necessary for enzyme induction was eliminated. The LD_{50} of EPN to rats treated with 50 mg/kg of phenobarbital 30 minutes prior to EPN was 6.7 mg/kg as compared with 7.3 for controls thus indicating no direct beneficial effect from phenobarbital treatment.

Discussion. Our recent observation(3) that the detoxification of the organophosphorus insecticide, EPN, is catalyzed by a microsomal enzyme system in the liver, stimulated interest in determining whether the known ability(1) of nikethamide to decrease the toxicity of EPN could be due to induced synthesis of this detoxification enzyme. If this were the case, other compounds having the ability to induce synthesis of microsomal enzymes, such as phenobarbital(5), should also decrease the susceptibility of rats to EPN. The ability of nikethamide to increase the rate of detoxification of EPN as demonstrated in this study provides an explanation for the protective action of this drug against EPN poisoning which was described by Di-Stefano *et al*(1). These investigators administered EPN and nikethamide at the same time by different routes along with atropine. It is probable that atropine prolonged survival of the animals for a sufficient period to allow induction of the detoxification enzyme to occur. The latent period observed by Rosenberg and Coon(2) in the action of nikethamide against EPN toxicity in rats is in agreement with this possibility. In the present study protection of EPN-treated animals was obtained without addition of atropine by pretreating the animals with nikethamide until maximum activity of the detoxification system was obtained. The ability of phenobarbital to induce an increase in the microsomal enzyme system that detoxifies EPN and to decrease the toxicity of EPN provides further evidence for the involvement of a microsomal enzyme in regulating the toxicity of EPN.

A direct pharmacological action by phenobarbital was excluded by the finding that when this drug was administered 30 minutes before EPN, to eliminate the latent period necessary for induction of hepatic microsomal enzymes and to achieve maximal pharmacological effects of phenobarbital at the time of administration of EPN, there was no reduction of the toxicity of EPN to rats.

The results of this study have indicated that measurement of the rate of detoxification of EPN by the livers of animals treated with various chemical agents provides a convenient method of measuring alteration of a microsomal enzyme system by drugs and other foreign chemicals. Results obtained with EPN and enzyme inducing agents should not, however, be extrapolated to other organophosphorus insecticides because the extent to which the detoxification of other insecticides of this class is dependent upon microsomal

enzyme systems has not yet been determined.

Summary. Administration of nikethamide or phenobarbital to rats increases the rate of degradation of the organophosphorus insecticide, EPN, by a microsomal oxidase system in the liver. Induction of the activity of this system by nikethamide provides a possible explanation for the previously demonstrated ability of this drug to decrease the susceptibility of animals to the acute toxicity of EPN. In the present study there was good correlation between the extent of increase in activity of the detoxification enzyme system by nikethamide and phenobarbital and the ability of these compounds to reduce the acute toxicity of EPN to rats with phenobarbital being superior to nikethamide in both respects. It appears that the microsomal enzyme system that catalyzes the degradation of EPN to p-nitrophenol provides a practical means of testing various chemical agents for ability to induce synthesis of microsomal enzyme activity.

1. DiStefano, V., Hurwitz, L., Neuman, W. F., Hodge, H. C., Proc. Soc. Exp. Biol. and Med., 1951, v78, 712.

2. Rosenberg, P., Coon, J. M., J. Pharm. Exp. Therap., 1960, v128, 289.

3. Neal, R. A., DuBois, K. P., ibid., 1965, v148, 185.

4. Brazda, F. G., Baucum, R., ibid., 1961, v132, 295.

5. Conney, A. H., Burns, J. J., Adv. Pharm., 1962, v1, 31.

6. Litchfield, J. T., Wilcoxon, F., J. Pharm. Exp. Therap., 1949, v95, 99.

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Human Growth Hormone Immunoassay: Two-Antibody Method Using I-125 Tracer.* (30698)

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Methods for immunoassay of human growth hormone using an I-131 tracer have been reported(1,2). Experience with insulin immunoassay by the 2-antibody technique has demonstrated that this procedure can be conveniently and reliably used(3-7). The present report describes a two-antibody procedure for immunoassay of human growth hormone utilizing a commercially prepared I-125 human growth hormone tracer.

Materials and methods. Each of 6 rabbits received a subcutaneous injection of 1 mg of human growth hormone† (HGH) on days 0, 7, 14, and 21. The HGH was prepared in a solution of 0.15 M NaCl, phosphate buffer (pH 7.4); the final concentration was 1 mg/ml. Equal volumes of HGH solution and lanolin-oil were mixed to a thick emulsion(8). After the 4 injections, each animal was bled *via* cardiac puncture on days 32 and 35, *i.e.*, at 11 and 14 days after the fourth injection. Two of the six rabbits responded with anti-human growth hormone (AHGH-R) serum which, at a final dilution of 1:4,000, was capable of binding approximately 50% of 0.5 mμg of I-125-HGH.

The I-125-HGH was prepared commercially.‡ The specific activity of the product was 390 millicuries per milligram.

Anti-rabbit serum (ARS-S) was obtained from sheep which had received subcutaneous injections of rabbit serum plus lanolin-oil adjuvant. One of three sheep responded with

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‡ Abbott Laboratories, Oak Ridge, Tenn. This was Andersen's HGH preparation, which was also used to immunize the rabbits.