a profound fall in urinary excretion of uric acid. C_{urate} declined from a mean control level of 10.4 ml/min. to a mean minimum of 1.4 ml/min. The uricosuria produced by salicylate and probenecid was temporarily abolished by lactate. The significance of these findings is discussed.

1. Gibson, H. V., and Doisy, E. A., *J. Biol. Chem.*, 1923, v55, 605.
2. Quick, A. J., *ibid.*, 1932, v98, 157.
3. Michael, S. T., *Am. J. Physiol.*, 1944, v141, 71.
4. Nichols, J., Miller, A. T., Jr., and Hiatt, E. P., *J. Applied Physiol.*, 1951, v3, 501.
5. Goldring, W., and Chasis, H., *Hypertension and Hypertensive Diseases*, The Commonwealth Fund, N. Y., 1944.
6. Sirota, J. H., Yü, T. F., and Gutman, A. B., *J. Clin. Invest.*, 1952, v31, 692.
7. Crosley, A. P., Jr., Brown, J. F., Schuster, B., Emanuel, D. A., Tuchman, H., Castillo, C., and Rowe, G. G., *J. Lab. and Clin. Med.*, 1957, v49, 429.
8. McDonald, R. K., Shock, N. W., and Yiengst, M. J., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 686.
9. Gutman, A. B., Yü, T. F., and Sirota, J. H., *Fed. Proc.*, 1956, v15, 85.
10. Yü, T. F., Berger, L., Stone, D. J., Wolf, J., and Gutman, A. B., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 264.
11. Harding, V. J., Allen, K. D., Eagles, B. A., and Van Wyck, H. B., *J. Biol. Chem.*, 1925, v63, 37.
12. Lockie, L. M., and Hubbard, R. S., *J.A.M.A.*, 1935, v104, 2072.
13. Adlersberg, D., and Ellenberg, M., *J. Biol. Chem.*, 1939, v128, 379.
14. Altschule, M. D., Perrin, G. M., and Holliday, P. D., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 446.
15. Unpublished studies.
16. Quick, A. J., *J. Biol. Chem.*, 1935, v110, 107.
17. Craig, F. N., *Am. J. Physiol.*, 1946, v146, 146.
18. Miller, A. T., and Miller, J. D., Jr., *J. Applied Physiol.*, 1949, v1, 614.

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Quantitative Measurement of Inhibition of the Enzymatic Detoxification of Malathion by EPN (ethyl p-nitrophenyl thionobenzenephosphonate).* (23617)

SHELDON D. MURPHY AND KENNETH P. DUBOIS
Department of Pharmacology, University of Chicago

Frawley *et al.*(1) recently demonstrated that simultaneous administration of ethyl p - nitrophenyl thionobenzenephosphonate (EPN) and S-(1,2-dicarbethoxyethyl)-O, O-dimethyl phosphorodithioate (malathion) to rats and dogs causes marked potentiation of the acute and subacute toxicity of these insecticides. The widespread use of organic phosphorus-containing insecticides on food crops makes it possible for the diet of man and domestic animals to contain low quantities of several of these compounds. For an accurate evaluation of the health hazards which might result from potentiation of the action of cholinergic organic phosphates it was desirable to have a sensitive, direct method for measuring the biochemical event responsible for the effect which could be applied to the tissues of animals fed low levels of these insecticides. It has been observed by Cook *et al.*(2) that EPN inhibits the hydrolytic detoxification of malathion by rat liver homogenates *in vitro*. This observation provides a possible explanation for potentiation of the toxicity of malathion by EPN since the low mammalian toxicity of malathion is apparently highly dependent upon rapid and complete detoxification. The present communication describes a quantitative method for measuring inhibitory action of EPN on detoxification of malathion. Data are presented which show the applicability of this method for *in vitro* experiments and for measurements on tissues taken from animals given acutely toxic and low dietary levels of EPN. The tissue distribution of the esterase which catalyzes the hydrolytic detoxification of malathion was also studied.

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Methods and materials. Adult male and

female Sprague-Dawley rats (250-400 g), male and female Carworth Farms mice (25-35 g) and adult male guinea pigs (*ca* 300 g) were used for this study. The rats and mice were fed Rockland Rat Diet and the guinea pigs received Rockland Rabbit Pellets. For experiments on the metabolism of malathion the animals were anesthetized with ether and blood was withdrawn by cardiac puncture. The animals were then sacrificed and the tissues were quickly removed, weighed and homogenized in cold water. Solutions of malathion or EPN (0.1 M) were prepared for the enzyme studies by dissolving the compounds in ethanol and diluting to the desired concentration with distilled water. For *in vivo* experiments solutions of EPN dissolved in 10% ethanol and 90% propylene glycol and undiluted malathion were given intraperitoneally. Chemical conversion of malathion to its oxygen analogue (malaoxon) was accomplished by a modification of the method of Fallscheer and Cook(3). Thus 2 ml of water and 3 ml of dilute bromine water (0.1 ml of saturated bromine water in 25 ml of distilled water) were added to 5 ml of 1 x 10^{-3} M technical malathion (95%). This solution was allowed to stand for one hour during which time complete oxidation of malathion occurred. The concentration of bromine present in the oxidized malathion solution did not interfere with the activity of the enzyme systems to which it was added. The bromine-treated malathion produced 50% inhibition of rat brain cholinesterase at a final molar concentration of 1.6 x 10^{-6}. Enzymatic conversion of malathion to malaoxon was accomplished by the method of Murphy and DuBois(4) using 1 x 10^{-4} M malathion as the substrate. Cholinesterase measurements were performed manometrically by the method of DuBois and Mangun(5). The amount of oxidized malathion produced or destroyed during incubation with tissue homogenates was measured by a bioassay procedure using the cholinesterase system in the manner which we described previously(4).

Results. To perform quantitative measurements of the inhibitory effect of EPN on the detoxification of malathion a method was needed for measuring the activity of the enzyme which catalyzes the hydrolytic detoxification of malathion and its toxic oxygen analogue (malaoxon) in animal tissues. A number of preliminary experiments on the optimum conditions for measuring the activity of the esterase resulted in the development of a procedure which satisfied the requirements of a quantitative enzyme assay. The oxygen analogue of malathion was employed as the substrate in the test system since it has strong anticholinesterase activity in contrast to pure malathion which has no action on this enzyme *in vitro*. It was possible to utilize the anticholinesterase activity of the oxygen analogue (malaoxon) to bioassay the quantity of malaoxon destroyed by the esterases of animal tissues. The test system contained 0.15 ml of 5 x 10^{-4} M malaoxon, 0.4 ml of 0.1 M phosphate buffer (pH 7.2), 0.1 or 0.2 ml of cold, aqueous tissue homogenate and enough distilled water to make a final volume of 3 ml. Assays were always performed in duplicate using 2 levels of tissue. Thus 0.1 ml and 0.2 ml of 2.5% liver (2.5 mg and 5 mg) and 10 and 20 mg of serum were used except in those experiments in which the inhibitory effect of EPN necessitated the use of higher quantities. The tubes containing all of the constituents of the reaction mixture except the substrate were placed in a constant temperature bath at 38°C and the malaoxon was then added. After incubation for 10 minutes aliquots (0.6 ml) were withdrawn and added immediately to the cholinesterase test system(5) containing 50 mg of homogenized rat brain as the source of the enzyme. From the amount of inhibition of cholinesterase produced by malaoxon before and after incubation with tissue homogenates it was possible to calculate the quantity of malaoxon which was detoxified by using the logarithmic relationship between inhibition and malaoxon concentration shown in Fig. 1.

In the test system described above the rate of detoxification of malathion was dependent upon the tissue concentration. Thus 2.5 and 5 mg of liver detoxified 9.1 and 20.1 μg of malaoxon respectively. Under the conditions selected for the assay the reaction rate was linear with time as evidenced by detoxification of 4.7, 8.9 and 13.7 μg of malaoxon by 2.5 mg

TABLE I. Detoxification of Malaoxon by Tissues of Various Species.

Tissue	μg of malaoxon destroyed/mg tissue/10 min.			
	Rats	Mice	Guinea pigs	Dogs
Liver	4.03 ± .17*	1.84 ± .15	3.74 ± .28	11.5 ± 1.1
Kidney	.66 ± .04	.46 ± .05	.21 ± .13	1.85 ± .66
Serum	.99 ± .07	1.78 ± .08	.19 ± .03	.08 ± .06
Lung	.75 ± .05	.58 ± .02	.19 ± .13	1.18 ± .11
Ileum	.39 ± .02	.23 ± .03	.13 ± .04	.07 ± .02
Spleen	.12 ± .03	.03 ± .01	.16 ± .12	.03 ± .03

* Figures represent avg values ± avg dev. from mean.

of liver in 5, 10 and 15 minutes respectively. Liver homogenates heated at 100°C for 2 minutes lost their ability to catalyze the detoxification of malaoxon and dialysis against distilled water for 18 hours at 5°C caused only 6% loss of enzyme activity. Storage of whole liver homogenates at 5°C for 18 hours caused only 10% loss of activity.

The first application of the method for measuring the detoxification of malaoxon consisted of a survey of the enzyme activity of tissues from various species. The results of these measurements are summarized in Table I in which each value is an average of assays on the tissues of at least 3 animals. These measurements demonstrated that liver exhibits the highest activity in all species used with dog liver being the most active. The serum of mice and rats was capable of detoxifying a considerable quantity of malaoxon but dog serum exhibited no activity. Kidney and lung also contained the detoxifying enzyme. Other assays conducted on female mice and rats indicated that there is no sex difference in the enzyme activity of the liver of mice and the serum of mice and rats. However, the activity of the liver of male rats was about 4 times as great as that of females as evidenced by the detoxification of 4 (range 3.4 to 4.5) μg of malaoxon/mg of male rat liver and 0.99 (range 0.9 to 1.1) μg/mg by the livers of female rats.

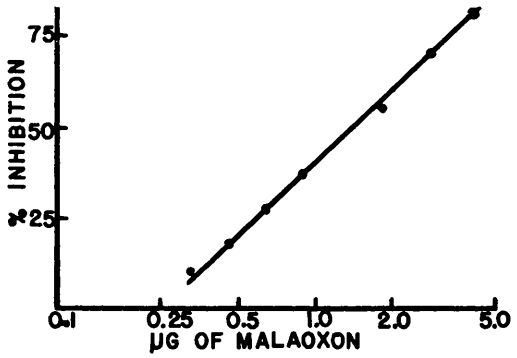

FIG. 1. Inhibitory effect of malaoxon on brain cholinesterase *in vitro*. Ordinate, % inhibition; abscissa, concentration of malaoxon plotted logarithmically.

Addition of EPN to the enzyme system which catalyzes the detoxification of malaoxon markedly inhibited the reaction. When various concentrations of EPN were incubated with homogenized liver for 5 minutes prior to addition of malaoxon the amount of inhibition of the detoxifying esterase was proportional to the logarithm of the EPN concentration within the range of EPN concentrations producing between 20% and 80% inhibition. In these tests 50% inhibition of the enzyme activity of male rat liver occurred with a final concentration of 2.8×10^{-5} M EPN and the lower activity of the liver of female rats was inhibited to the same extent by 5×10^{-6} M EPN. To ascertain whether this inhibitory effect also occurs *in vivo* a group of 3 adult male rats was given one-half of the LD_{50} of EPN (13 mg/kg) intraperitoneally and one hour later the animals were sacrificed for measurements of the enzyme activity of serum and liver. Complete inhibition of the ability of the serum and liver to detoxify malaoxon was observed. This finding was followed by similar experiments in which several lower doses of EPN were tested to find the quantity which produced partial inhibition of the detoxification enzyme. The results summarized in Fig. 2 represent the average of assays performed on tissues from groups each containing at least 3 male rats which received 0.5, 1 or 1.5 mg/kg of EPN intraperitoneally. The per cent inhibition of enzyme activity was calculated from the assay results using the normal values shown in Table I as a basis for the

calculations. The data in Fig. 2 show that 84% and 93% inhibition of the ability of liver and serum of male rats to detoxify malaoxon occurred in 1 hour after the administration of 1.5 mg/kg of EPN. Significant inhibition was also observed with 0.5 mg/kg of EPN which is approximately one-fiftieth of the LD_{50}. The effect of inhibition of the detoxifying esterase by EPN on the susceptibility of rats to the acute toxicity of malathion was demonstrated by determining the LD_{50} of malathion to male rats treated 1 hour previously with 1.5 mg/kg of EPN. The approximate LD_{50} of malathion to these animals was 550 mg/kg as compared with 1100 mg/kg for comparable controls. The dose of EPN used in this experiment did not cause any inhibition of the cholinesterase activity of brain.

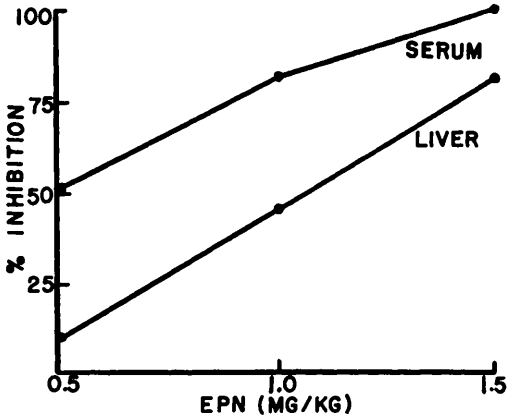

FIG. 2. Inhibitory effect of EPN *in vivo* on ability of rat tissues to detoxify malaoxon. Assays performed on tissues taken from rats 1 hour after intraperitoneal administration of EPN.

serum or submaxillary glands and the increased toxicity of malathion can, therefore, be attributed to inhibition of its normal detoxification.

The duration of the inhibitory effect of EPN was studied by administering 1.5 mg/kg of EPN to a series of male rats and sacrificing them at various intervals for assays on serum and liver. The results of these measurements are shown in Fig. 3 in which each value on the curves represents an average for the tissues of 3 animals. Slow reversal of the enzyme inhibition was observed as evidenced by a gradual return of the activity during a 72-hour observation period.

TABLE II. Effect of Feeding EPN on the Ability of Rat Tissues to Detoxify Malaoxon.

Dietary level of EPN (ppm)	μg of malaoxon destroyed/mg tissue/10 min.		% inhibition	
	Liver	Serum	Liver	Serum
Control	5.6 ± .72	1.52 ± .23		
5	4.0 ± .15	.92 ± .10	28.6	39.5
10	2.4 ± .5	.62 ± .18	57.1	59.2
20	1.2 ± .15	.29 ± .12	78.6	80.9
50	.62 ± .09	.13 ± .02	87.9	91.4
100	.28 ± .13	.07 ± .03	95.0	95.4

It was considered important to ascertain the effects of diets containing EPN on the activity of the enzyme which detoxifies malaoxon since EPN is used as an insecticide on food crops. For this experiment groups each containing 4 male rats were fed various levels of EPN in the diet for 2 weeks and were then sacrificed for measurement of the ability of liver and serum to detoxify malaoxon. The results of these assays are summarized in Table II where it may be seen that levels as low as 5 ppm of EPN caused inhibition of the detoxification enzyme. The amount of inhibition was dependent upon the dietary level of EPN over the range of 5 to 100 ppm of EPN with nearly complete inhibition being observed at the highest level.

The inhibitory action of EPN on the detoxification of certain cholinergic organic phosphates has a useful application in research on the enzymatic conversion of thiophosphates to their corresponding oxygen ana-

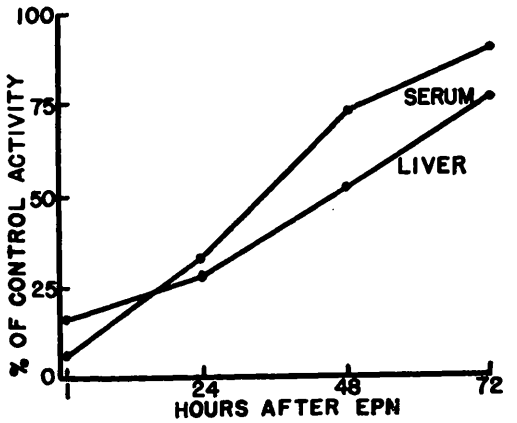

FIG. 3. Duration of inhibitory effect of a single intraperitoneal dose of EPN (1.5 mg/kg) on ability of rat tissues to detoxify malaoxon.

logues. In a previous communication(4) we described experiments which demonstrated that the enzymatic conversion of thiophosphates to their oxygen analogues *in vitro* is complicated by coincident hydrolytic destruction of the compounds and their active metabolites. The strong inhibitory action of EPN on the detoxification of malathion suggested the use of EPN to inhibit destruction during studies on the enzymatic conversion of malathion to malaoxon by liver homogenates. When malathion (1×10^{-4} M) was incubated with 2.5 to 25 mg of whole liver homogenate using the test system described by Murphy and DuBois(4) no accumulation of malaoxon occurred in the reaction mixture. However, homogenates prepared from the livers of 5 male rats which were sacrificed at 1 hour after intraperitoneal administration of 13 mg/kg of EPN produced an average of 1 μg of malaoxon/mg of liver/10 minutes. The results of this experiment demonstrated that the conversion of malathion to its cholinergic oxygen analogue can be studied *in vitro* using whole liver homogenates by inhibiting the enzyme responsible for the breakdown of the parent compound and its active metabolite with EPN. It seems likely that this same procedure can be employed for studying the metabolic conversion of some of the other insecticidal thiophosphates to toxic metabolites.

Discussion. The present investigation resulted in the development of a quantitative method for measuring the enzymatic detoxification of malathion and its active metabolite, malaoxon, by mammalian tissues. A survey of tissues from various species revealed that liver, serum, kidney and lung contained the highest concentrations of the enzyme with the exceptions being very low activity in the serum of guinea pigs and dogs and the lungs of guinea pigs. In the present study no attempt was made to identify the degradation products of malathion or malaoxon but other investigators(2,6,7) have presented evidence that the detoxification consists of hydrolysis of the ester linkages in the 1,2-dicarbethoxyethyl side-chains. It seems reasonable to assume that the esterase which catalyzes this reaction may function in the detoxification of various ester-type drugs and food constituents.

The marked inhibitory action of EPN on the detoxification of malaoxon *in vitro* is in agreement with the findings of Cook *et al.*(2) and provides an explanation for the ability of EPN to potentiate the toxicity of malathion. More direct evidence in support of this possibility was obtained in the present study by the parenteral administration and feeding of EPN to rats. Single doses of EPN, below those which affect cholinesterase activity, markedly inhibited the ability of serum and liver to detoxify malaoxon and greatly increased the toxicity of injected malathion. The feeding of diets containing quantities as low as 5 ppm of EPN for 2 weeks caused significant inhibition of the detoxification enzyme. This enzyme system is thus considerably more sensitive toward inhibition by EPN than is cholinesterase because the lowest dietary level of EPN which produces significant inhibition of cholinesterase in a period of 2 weeks has been found(8) to be 25 ppm. On the basis of our finding that reversal of inhibition of the detoxifying esterase is relatively slow EPN may exert a cumulative toxic effect on this enzyme when fed in the diet for prolonged periods of time. Since inhibition of the enzyme was demonstrated by feeding levels of EPN near the maximum amount (3 ppm) which is currently permitted as a residue in food crops further studies are indicated to determine the influence of dietary EPN on the detoxification of various organic phosphates and drugs containing ester linkages.

Summary. A quantitative method for measuring the hydrolytic detoxification of malathion and its toxic metabolite, malaoxon, was developed. Application of the method to normal tissues revealed that the liver of several species contains the highest concentration of the enzyme but serum, kidney and lung also exhibited activity in some species. EPN was found to inhibit the enzyme system which detoxifies malathion *in vitro* and *in vivo*. This interference with the detoxification of malathion by EPN provides an explanation for the potentiation of toxicity which occurs when the two compounds are administered simultaneously and suggests the possibility that EPN may inhibit the detoxification of other esters which utilize the same detoxification

pathway.

1. Frawley, J. P., Hagan, E. C., Fitzhugh, O. G., Fuyat, H. N., and Jones, W. I., *J. Pharm. and Exp. Ther.*, 1957, v119, 147.

2. Cook, J. W., Blake, J. R., and Williams, M. W., *J. Assn. Off. Agric. Chem.*, 1957, v40, 664.

3. Fallscheer, H. O., and Cook, J. W., *ibid.*, 1956, v39, 691.

4. Murphy, S. D., and DuBois, K. P., *J. Pharm. and Exp. Ther.*, 1957, v119, 572.

5. DuBois, K. P., and Mangun, G. H., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 137.

6. March, R. B., Fukuto, T. R., Metcalf, R. L., and Maxon, M. G., *J. Econ. Entomol.*, 1956, v49, 185.

7. O'Brien, R. D., *ibid.*, 1957, v50, 159.

8. Frawley, J. P., Hagan, E. C., and Fitzhugh, O. G., *J. Pharm. and Exp. Ther.*, 1952, v105, 156.

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Isolation of Nutritional Variants from Conjunctival and HeLa Cells.* (23618)

R. SHIHMAN CHANG (With technical assistance of Helen Liepins)
Department of Microbiology, Harvard School of Public Health, Boston

Properties acquired by human cells upon prolonged cultivation have been a subject of considerable interest. Attention, however, has been directed chiefly toward the problem of acquired malignancy as evidenced by the numerous publications on this subject(1). While changes in the nutritional requirements of various laboratory cell strains have long been suspected, a report on the appearance of a variant capable of utilizing a chemical compound in lieu of an essential nutrient to support growth has been conspicuously lacking. Those of Haff and Swim(2), Puck(3) and Chang(4) referred to quantitative differences in the requirement of, or tolerance to, certain biologicals of unknown complex composition. This publication describes the successful isolation of nutritional variants from our cultures of human conjunctival(5) and HeLa cells(6). These variants are capable of utilizing certain chemical compounds in lieu of glucose to support continuous cell growth.

Material and methods. The maintenance of stock cell cultures; collection, storage and dialysis of serum; composition of the basal carbohydrate-free medium; glucose oxidase test; enumeration of cell number, and, assessment of cell multiplication rate have already been described(7). *Compounds.*† D(+)xylose, d-ribose, d-arabinose, sodium lactate, sodium pyruvate and alanine were used. These compounds were tested for possible trace contamination with glucose by the glucose oxidase test, and for their ability to prevent progressive degeneration of the stock conjunctival cells at concentrations of 125, 25 and 5 mM in the basal media. With the exception of d(+)xylose, these compounds gave negative glucose oxidase tests at concentrations of 2M, and were unable to prevent progressive cell degeneration. The three preparations of d(+)xylose tested gave positive glucose oxidase reactions at concentration of 0.1 to 0.2 M, and 2 of them were able to prevent progressive cell degeneration at concentrations of 25 and 125 mM. Although treatment of these xylose preparations with glucose oxidase, as described(7), failed to reduce appreciably their reactivities in the glucose test,‡ their ability to prevent cell degeneration was abolished. Thus, d(+)xylose which has been pretreated with glucose oxidase was used exclusively in this study. *Isolation of variants.* About one million cells

* Supported in part by grants from N.I.H., and Amer. Cancer Soc.

† D(+)xylose, C. P., was purchased from Pfanstiehl, Fisher and Nutritional Biochemicals; d-ribose, d-arabinose and sodium pyruvate, from Nutritional Biochemicals; sodium lactate, from Mallinckrodt; and dl alanine, from Eastman Kodak.

‡ Slow oxidation of d(+)xylose in the presence of glucose oxidase has been described(8).