Effect of Tolbutamide on the Metabolism of Parathion¹⁾

EIGO TAKABATAKE, TOSHIHIKO ARIYOSHI, and KATSUHIRO SCHIMIZU

Faculty of Pharmaceutical Sciences, Nagasaki University²⁾

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Effect of the pretreatment with tolbutamide in female rats on the liver microsomal activation (anticholinesterase activities) and degradation (formation of *p*-nitrophenol) of parathion was studied. Both pathways were stimulated by tolbutamide-pretreatment, but the degradation process was superior to the activation of parathion at lower concentration of substrate in the liver preparation of rats pretreated with tolbutamide. The formation of *p*-nitrophenol from paraoxon by liver homogenate was also accelerated by tolbutamide. These facts should be reflected in the lowering of parathion toxicity in animals pretreated with tolbutamide.

The lowering of toxicity of parathion in mouse or dog was observed following the administration of tolbutamide and such effect of some tolbutamide analogues has been compared³⁾. On the other hand, Remmer, *et al.*⁴⁾ showed that tolbutamide was a potent inducer for the drug metabolizing enzyme in liver microsomes. Furthermore, some chlorinated insecticides or chlorcyclizine, also inducing drug metabolism, have been reported to lower the toxicity of organophosphate anticholinesterases.^{5,6)}

Parathion must undergo oxidative desulfuration in order to inhibit cholinesterase and hydrolytic cleavage for detoxication. Nakatsugawa and Dahm⁷⁾ using ³⁵S-parathion and Neal⁸⁾ using ³²P-parathion recently showed that both activation and degradation of parathion were catalyzed by microsomal enzyme depending to NADPH and O₂ as is seen in the metabolism of various drugs. If tolbutamide would stimulate only the activation process, the toxicity of parathion should be increased. If this drug would stimulate more the detoxication process, the toxicity of parathion could be decreased. This work was attempted to examine which way would be more stimulated by tolbutamide.

Experimental

Female rats of Wistar-King strain, 80-120 g, and male mice of ddN strain, about 15 g, were used.

Chemicals—Parathion, kindly provided by the Ihara Noyaku Co., was dissolved in ether and washed with 10% Na₂CO₃, then ether solution dried with anhydrous Na₂SO₄ was evaporated *in vacuo*. Purified parathion was dissolved in propyleneglycol at 40 μmole/ml as stock solution and diluted with same solvent just before use. Paraoxon, kindly provided by Sumitomo Chemical Co., was used as same as parathion.

Pretreatment—Tolbutamide was suspended in 1M NaHCO₃ and given to rats *per os* at a dose of 75 mg/0.5 ml/100 g once a day for 3 days. In the case of mice, tolbutamide dissolved in propyleneglycol was subcutaneously injected 750 mg/kg once a day for 7 days.

Parathion Metabolizing System—Liver 9000 g supernatant fraction were prepared by homogenation with 2 vol of 0.1 M phosphate buffer, pH 7.4, containing 75 mM of nicotinamide and centrifugation at

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- 7) T. Nakatsugawa and P.A. Dahm, *Biochem. Pharmacol.*, **16**, 25 (1967).
- 8) R.A. Neal, *Biochem. J.*, **103**, 183 (1967).

9000 g for 20 minutes in cold. An incubation mixture contained 2 ml of 9000 g supernatant, 50 μ moles of $MgCl_2$, 10 μ moles of glucose-6-phosphate, 0.5 μ moles of NADP, 0.5 ml of parathion solution in propyleneglycol and 0.1 M phosphate buffer making total volume to 5 ml. Incubation was performed at 37°.

Anticholinesterase Activity—At appropriate time an aliquot of incubation mixture was taken and diluted with water to 200–400 fold. To a mixture of 0.5 ml of 10% rat brain homogenate in 1.15% KCl and 1.0 ml of 0.1 M Tris, pH 7.5, was added 0.5 ml of diluted sample and kept at 37° for 1 hr. Instead of sample, 0.5 ml of water was added for a blank run and 0.5 ml of 1% neostigmine methylsulfate for a control run. To the incubated mixture, was added 1.0 ml of 0.2% acetylcholine-HCl and incubation was continued for further 40 minutes. Remaining acetylcholine was determined colorimetrically. Percent inhibition of cholinesterase was calculated as following,

$$\text{Percent Inhibition} = (\text{O.D. of sample} - \text{O.D. of blank}) / (\text{O.D. of control} - \text{O.D. of blank}) \times 100$$

Serum cholinesterase activity was also colorimetrically determined using 1 ml of 10 fold diluted serum.

p-Nitrophenol—To 2.5 ml of incubation mixture, was added 0.5 ml of HCl and extracted with 30 ml of 1% *n*-AmOH in ether-petroleum ether (1:4). After reextraction of 20 ml of solvent phase with 4 ml of 2 N NH_4OH , ammonia solution was mixed with 0.5 g of zinc powder and centrifuged. To the supernatant solution, was added 0.5 ml of 1% *o*-cresol and absorbance was measured at 620 $m\mu$ after standing for 1 hr.

Liver and Plasma A-Esterase—The formation of *p*-nitrophenol from paraoxon by liver homogenate and plasma was determined colorimetrically according to Toriolo and Coon.⁶⁾

Results

Toxicity of Parathion—As shown in Table I, mortality of mice by parathion was lower in tolbutamide-group than control below 13.4 mg/kg but mortality of both group was same at 16.0 mg/kg.

TABLE I. Mortality of Mice by Parathion

Dose of Parathion (mg/kg)	Number of Death/Number of Test	
	Control	Tolbutamide
6.41	4/7	0/5
7.70	3/7	1/7
9.33	5/7	0/6
13.40	5/7	3/7
16.00	7/7	6/6

Plasma Cholinesterase Activity—Plasma cholinesterase activity of control rats decreased to 60 % of normal value at 6 hr after administration of 6 mg/kg of parathion, but that of tolbutamide treated rats still remained at 85% (Table II).

TABLE II. Plasma Cholinesterase Activities of Mice after Parathion Administration

Time after parathion injection (6 mg/kg) (hr)	Relative plasma cholinesterase activity	
	Control	Tolbutamide
Before	100	100
3	74	93
6	60	85

Activation of Parathion—The time course of activation of parathion by liver 9000 g supernatant fraction was shown in Fig. 1. In the case of the liver of rats treated with tolbutamide, anticholinesterase activity has quickly reached to a peak and rapidly reduced. On the contrary, control liver preparation activated parathion more slowly but maintained high level longer.

Table III shows the anticholinesterase activity of diluted incubation mixture of parathion with liver 9000 g supernatant at different time and various concentrations of substrate. At 5 minutes of incubation period, anticholinesterase activities of tolbutamide-group were higher than control except lowest concentration of parathion, 2.5×10^{-5} M. At 30 and 60 min, anticholinesterase activities of tolbutamide-group were lower than control except at high concentration of parathion, 2 or 4×10^{-4} M. Comparing at 30 and 60 min in these concentrations of substrate, control group showed almost same activities but tolbutamide-group apparently reduced antienzyme activities during this 30 min. These data suggested that the activation of parathion by liver preparation was stimulated by the pretreatment with tolbutamide and degradation of toxic material was also accelerated. Thus, remaining amount of toxic material was less in tolbutamide-group than control at relatively low concentration of parathion. However, at higher concentration of substrate, the activation process was superior to degradation.

Fig. 1. Time Course of Activation of Parathion by Liver 9000 g Supernatant of Rats of Control or Pretreated with Tolbutamide

○ Control; × Tolbutamide
Substrate concentration: 2×10^{-5} M

TABLE III. Effect of Tolbutamide Pretreatment on the Activation of Parathion by Liver 9000 g Supernatant

Parathion concentration $\times 10^5$ M	Per cent inhibition of cholinesterase					
	5 min		30 min		60 min	
	Control	Tolbutamide	Control	Tolbutamide	Control	Tolbutamide
2.5	23.3	22.7	28.1	15.0	9.6	8.4
5.0	27.6	40.0	37.1	29.9	16.8	14.4
10.0	35.0	60.0	59.3	50.8	35.3	25.1
20.0	36.2	65.3	68.2	86.2	65.2	74.8
40.0	48.5	77.2	76.6	92.7	74.2	76.6

Anticholinesterase activities were determined by 400-fold diluted samples.

***p*-Nitrophenol from Parathion**—As shown in Table IV, the formation of *p*-nitrophenol from parathion by liver preparation was higher in tolbutamide-group than control at any concentration of substrate. Lineweaver-Burk plot gave almost same value of K_m , 6.25×10^{-4} M, for both groups and V_m for control 294 $\mu\text{mole/g/hr}$ and for tolbutamide-group 665 $\mu\text{mole/g/hr}$.

TABLE IV. Effect of Tolbutamide Pretreatment on the Formation of *p*-Nitrophenol from Parathion by Liver 9000 g Supernatant

Parathion Concentration $\times 10^4$ M	<i>p</i> -Nitrophenol formed, $\mu\text{mole/g/hr}$	
	Control	Tolbutamide
2.0	68	177
3.0	99	215
4.0	119	230
8.0	171	285
16.0	246	363

Liver and Plasma A-Esterase (*p*-Nitrophenol from Paraoxon)—Table V shows that tolbutamide-pretreatment stimulated the formation of *p*-nitrophenol from paraoxon by liver and depressed that by plasma. This difference was found at high concentration of paraoxon, 20 mg in 10 ml.

TABLE V. Effect of Tolbutamide Pretreatment on Liver and Plasma A-Esterase in Rat

	<i>p</i> -Nitrophenol formed, μ mole/g/hr	
	Control	Tolbutamide
Liver homogenate	3.76	5.28
Plasma	2.16	1.28

The *p*-nitrophenol liberated by 0.5 ml of liver homogenate or plasma from 10 ml of the substrate solution containing 20 mg of paraoxon was measured.

Sex Difference in Parathion Metabolism—As shown in Table VI, both activation and degradation of parathion were higher in male rat than female. Castration in male markedly reduced metabolism of parathion, either.

TABLE VI. Sex Difference in Parathion Metabolism

Sex	Treatment	Percent inhibition cholinesterase ^a	<i>p</i> -Nitrophenol formed (m μ mole/g/hr)
Male	Control	100	540
	Castration 2 week		323
Female	Control	41	137
	Tolbutamide	84	262

^a) Assayed using 500-fold diluted samples which incubated with 8×10^{-4} M of parathion at 30 min.

Discussion

Organophosphate insecticides including parathion must undergo oxidative desulfuration in order to inhibit cholinesterase and hydrolytic cleavage to be detoxicated. Thus, the toxicity of these compounds are dependent on a balance of these pathways. Parathion is more toxic to female rats than to male rats but the conversion to paraoxon by the livers of male rats is considerably higher than that by female rats⁹ (Table VI). Neal and DuBois¹⁰ showed that the livers of adult male rats degraded O-ethyl O-(4-nitrophenyl) phenylphosphonothioate (EPN) and related phosphorothioates to phenols more rapidly than the livers of female or weanling male rats and that the rate of detoxication of these compounds is correlated with the *in vivo* susceptibility of animals to their toxicity. Castration in male rats reduced this pathway as shown in Table VI.

Nagase³ found the lowering effect of tolbutamide on the toxicity of parathion in mouse or dog and Remmer, *et al.*⁴ showed tolbutamide as a potent inducer of drug metabolizing enzyme. It is well known that the pharmacological activity or toxicity of a drug is regulated by its metabolism *in vivo* which is affected by the pretreatment with various drugs or chemicals. It is reasonable to expect that tolbutamide stimulates the metabolism of parathion and lowers the toxicity, but it was not clear how each process contributed to the overall toxicity.

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The magnitude of microsomal degradation of parathion has been reported to be more extensive than that of activation^{7,8)} and the pretreatment with tolbutamide increased the production of *p*-nitrophenol from parathion giving about two times high V_m value. Therefore, it might be reasonable to correlate the increase of *p*-nitrophenol production with the lowering toxicity of parathion. However, this assumption seems to be too simple because tolbutamide accelerated the activation of parathion, too. If the dose of parathion would be considerably high and the amount of paraoxon produced in a body would exceed the threshold at which animal would be immediately killed, the toxicity could not be decreased even if the degrading process of parathion would be increased to some extent by tolbutamide.

As shown in Table III, anticholinesterase activity obtained by liver preparation of tolbutamide-group were higher than that of control at any incubation time if the concentration of substrate was high. Antienzyme activities which were determined in this experiment corresponded just to remaining amount of paraoxon at a moment but not to the total production. The time course of parathion metabolism at low concentration indicated that both activation and degradation of parathion by liver of rats pretreated with tolbutamide were more rapid than control and integrated amount of toxic substance in the body of those rats might be less.

According to Nakatsugawa and Dahm,⁷⁾ paraoxon is not a substrate for the enzyme activating or degrading parathion in liver microsomes. Since the liver preparation used in the experiments of this report was not microsomes but 9000 g supernatant, it probably contained other enzymes and the amount of *p*-nitrophenol determined was not only from direct hydrolysis of parathion but also *via* paraoxon. When low concentration of paraoxon which would be produced in the activation system of parathion was tried to examine the enzymic degradation of paraoxon, it was difficult to find any difference between control and tolbutamide-pretreatment because of large spontaneous degradation of paraoxon in assay medium, pH 7.4. However, if high concentration of paraoxon was tried, the formation of *p*-nitrophenol from paraoxon by liver preparation was apparently higher in tolbutamide-group than control. A-esterase detoxicating paraoxon was increased in liver but decreased in plasma by tolbutamide as in the case of aldrin.⁶⁾ These facts are probably able to explain the lowering toxicity of parathion in animals pretreated with tolbutamide.

Neal⁸⁾ postulated the presence of two separate enzyme systems for the activation and degradation of parathion. The kinetic studies of each enzymes participating in metabolism of parathion will give additional information about the stimulatory effect of tolbutamide. Furthermore, the role of other factors involving the central nervous system which were pointed out by Triolo and Coon⁶⁾ also remained to be investigated.

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