Inhibition and Regeneration of Rat Liver Enzymes Hydrolyzing Acetanilide and *o*-Nitrophenyl Butyrate

F. Iverson
Bureau of Chemical Safety
Foods Directorate
Health Protection Branch
Ottawa, Canada, K1A 0L2

INTRODUCTION

Rat liver carboxylesterase has received considerable attention because of its ability to hydrolyse a carboxy ester bond of the insecticide Malathion. The enzyme is also susceptible to inhibition by the activated organophosphorous insecticides to the extent that loss of activity has been used as a measure of the 'no effect' levels of these compounds. (DUBOIS et al, 1968).

Classically, carboxylesterase activity has been differentiated from cholinesterase through the use of quaternary carbamate compounds, eg. physostigmine, which inhibit the latter, but not the former activity. Subsequent inhibition by organophosphates, such as DFP, will reveal arylesterase activity, which is not affected by either the carbamate or organophosphate inhibitors.

Recently, ARNDT and KRISCH (1972) suggested that rat liver amidase activity resulted from a series of carboxylesterases with overlapping substrate specificities. The amidase activity is toxicologically important since it is responsible for the hydrolysis of pharmaceuticals such as acetanilide and phenacetin. (HEYMANN et al., 1969).

Bulletin of Environmental Contamination & Toxicology,
Vol. 18, No. 4 © 1977 by Springer-Verlag New York Inc.

Other work (HAMILTON et al. 1975) has suggested that the differing substrate specificities of purified carboxylesterase fractions may be a reflection of contamination by other enzymes. A discussion of the problems associated with classification of carboxylesterases and the differentiation of carboxylesterase and amidase activity forms part of a review by JUNGE and KRISCH (1975). In view of the significance of carboxylesterase and acetanilide amidase activities, the present study was initiated to determine the suceptibility of these enzyme activities to inhibition by organophosphate and carbamate insecticides. O-nitrophenyl butyrate (ONPB) was selected as the ester substrate representative of carboxylesterase activity and acetanilide, a widely used analgesic, was chosen as an amide substrate. Since carbamylated esterases undergo a regeneration phenomenon, it was important to ascertain the ability of these enzymes to regenerate, and avoid any potentially harmful interactions between similar insecticides and pharmaceuticals. Although the regeneration of carbamylated acetylcholinesterase is rapid ($t_{\frac{1}{2}} \approx 30$ min at 25°C, REINER, 1971) data on other esterases is lacking, and it is only recently that REINER, (1971) determined that serum cholinesterase regenerated with a considerably longer half-life of approximately 3 hours (25°C).

METHODS

Male Sprague Dawley Rats 200 ± 20 gm obtained from Bio-Breeding, Ottawa, Canada, were housed in communal banks with free access to food and water. The rats were acclimatized for five days following which, each insecticide (analytical standard grade) was administered ip (1.0 ml/kg in distilled water) at one quarter its LD_{50}. In cases of insolubility, the compounds were dissolved in 50% ethanol w/v. The following dosages were administered to groups of 3 animals. Malathion; O,O-dimethyl phosphorodithioate ester of diethylmercaptosuccinate, 85 mg/kg. Parathion; O,O-diethyl O-p-nitrophenyl phosphorothioate, 1 mg/kg. Diazinon O,O-diethyl O-(2-isopropyl-6-methyl-4-pyrimidinyl)phosphorothioate 12 mg/kg. Guthion, O,O-dimethyl S-(4,oxo-1,2,3,-benzotriazin-3 [4H]-yl) methyl phosphorodithioate, 1 mg/kg. Sumithion, O,O-dimethyl O-(4-nitro-m-tolyl) phosphorothioate, 20 mg/kg. Methomyl, S-methyl-N-[(methylcarboxyl)oxy] thioacetimidate, 2 mg/kg. Carbaryl, 1-naphthyl N-methylcarbamate, 16 mg/kg. Temik, 2-methyl-2-(methylthio)propionaldehyde, O-(methylcarbamoyl) oxime, 0.25 mg/kg. Mobam, 4-benzothienyl N-methylcarbamate 11 mg/kg. Baygon, 2-isopropoxyphenyl N-methylcarbamate, 8 mg/kg. Controls received vehicle only.

Thirty minutes after dosing, the animals were sacrificed and the liver excised and quickly homogenized in 9 volumes ice-cold 50 mM Tris HCl buffer, pH 7.4. The homogenate was centrifuged at 9,000 x g for 20 minutes and the supernatant used for assay. When carbaryl was dosed, the supernatants were assayed for a 6 hour period at 25° to determine *in vitro* regeneration. Additional animals, in groups of 2, were treated with carbaryl and sacrificed at various times to determine the *in vivo* regeneration of enzyme activity.

In all cases, carboxylesterase activity was measured at pH 6.3 using o-nitrophenyl butyrate (ONPB) as substrate (MAIN and BRAID 1962). Amidase activity was measured using acetanilide according to BRODIE and AXLEROD (1948). The relative enzymatic activities reported were corrected for protein concentration as determined by the Biuret method of GORNALL et al. (1949).

RESULTS

The inhibition obtained with both substrates is shown in Table 1. The ratio of ONPB to acetanilide activity varied from 0.3 to 3.3 suggesting that both activities did not result from a single enzyme site.

The recovery of activity *in vivo* and *in vitro* after carbaryl administration is shown in figure 1. *In vivo*, both enzyme activities return to the control level in 6 hours. Although the data is generated from a crude system, an estimate of half-life for regeneration may be made from the data in figure 1. *In vitro* at 25° the half-life is approximately 6.5 hours. *In vivo*, the return of activity for both substrates is consistent with a half-life of approximately 1.5 hours.

Attempts to follow the *in vitro* regeneration of amidase activity resulted in significant loss of enzyme activity. An activity-time curve at 25°C for both substrates is shown in Figure 2, and reveals that the acetanilide amidase activity declines rapidly during the first hour to 50% of the control value.

DISCUSSION

Previous work (DUBOIS et al., 1968) has shown that arylamidase and carboxylesterase activites of rat liver homogenates are sensitive indicators of exposure to organophosphate insecticides. In the present study, the selected inhibitors lowered the enzymatic activity towards ONPB and acetanilide although for 4 of the organophosphate compounds the decrease in acetanilide amidase activity was marginal.

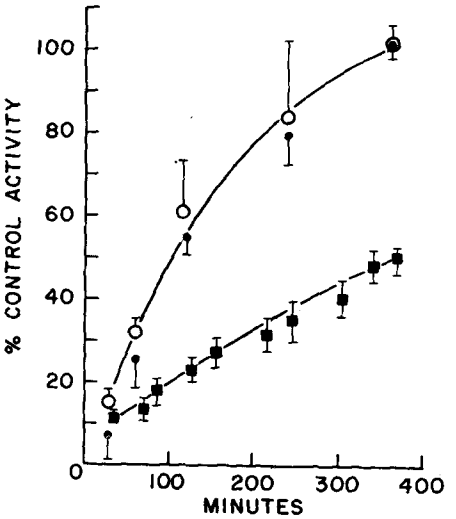

Figure 1. The in vitro and in vivo regeneration of acetanilide and o-nitrophenyl butyrate activity, after ip administration of carbaryl. (o). ONPB in vivo; (●) acetanilide, in vivo. Each point represents the mean and S.E. of 4 animals (■) ONPB in vitro, 25°C. Each point represents the mean of duplicate determinations from 3 rats.

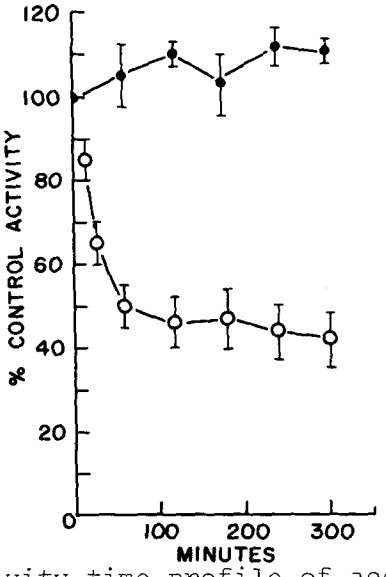

Figure 2. Activity-time profile of acetanilide and o-nitro-phenyl butyrate activities in 9,000 x g rat liver supernatant pH 7.4, 25°C. (●) ONPB; (o) acetanilide. Each point represents the mean and standard error (bar) from duplicate determinations of homogenates from livers of 3 rats.

TABLE 1

The inhibition of acetanilide and o-nitrophenyl butyrate activity in 9,000 x g supernatant of rat liver 30 min after dosing with $\frac{1}{4}$ LD_{50} of the insectidies listed. The values indicate the mean ±S.E. from 3 treated and 3 control rats.

Inhibitor	Acetanilide % Control	ONPB % Control	ONPB % Control/ Acetanilide % Control
Diazinon	19.4± 8.2	32.1± 7.1	1.7
Guthion	98.8±30.2	73.5±16.5	0.8
Sumithion	96.3± 3.2	29.6± 4.5	0.3
Malathion	96.1± 1.4	77.0± 4.2	0.8
Parathion	99.4±26.1	55±16.2	0.6
Methomyl	19.8± 7.4	57.8±10.1	2.9
Temik	33.0± 2.6	85.6±26.3	2.6
Baygon	14.1± 5.5	46.2± 6.9	3.3
Mabam	15.8± 2.5	4.7± 0.1	0.3
Carbaryl	11.2± 2.6	7.6± 1.3	0.7

The ratio of ONPB to acetanilide activity, differed from unity with each inhibitor. This suggests that there must be at least 2 distinct enzymes (or active sites) hydrolysing the different substrates. This was also evident from the observations that acetanilide amidase activity decreased to 50% of control values within one hour at 25°C, while ONPB activity was unchanged. The biphasic nature of the activity loss in turn suggests that at least 2 different enzymes are responsible for the hydrolysis of acetanilide.

The temperature sensitivity of the amidase activity is consitent with the report by PAHLICH and KRISCH (1969) who showed that with pig liver esterase preparations, the monoethylgycine 2,6-xylidide amidase activity was lowered to 9% of control values after 1 hour at 70°C while ONPB activity was reduced to 31% of

control levels. It would appear from the present study that a more pronounced difference occurs between acetanilide amidase and ONPB activity, at a significantly lower temperature.

Although the inhibitor and temperature sensitivities of the two substrate activities were different, the in vivo regeneration after carbaryl administration was similar, with both substrate activities returing to control levels within 6 hours. Therefore it would seem that only the most acute interactions between an N-methyl carbamate, such as carbaryl, and acetanilide would be of toxicological significance. The 6.5 hour half-life for regeneration at 25°C indicates that in this respect, carboxylesterase activity is more closely related to serum cholinesterase ($t_{\frac{1}{2}}$ ~ 3 hr) than erythrocyte acetylcholinesterase ($t_{\frac{1}{2}}$ ~ 30 min).

REFERENCES

ARNDT, R., and K. KRISCH. Z. Physiol. Chem. 353 589, (1972).

BRODIE, B.B., and J. AXLEROD. J. Pharmacol. Exp. Ther. 94 22, (1948).

DUBOIS, K.P., F.K. KINOSHITA, and J.P. FRAWLEY. Toxicol. Appl. Pharmacol. 12 273, (1968).

GORNALL, A.G., C.J. BRADAWILL, and M.M. DAVID. J. Biol. Chem. 177 751, (1949).

HAMILTON, S.E., H.D. CAMPBELL, J. de JERSEY, and B. ZERNER. Biochem. Biophys. Res. Comm. 63 1146, (1975).

HEYMANN, E., K. KRISCH., H. BUCH, and W. BUZELLO. Biochem. Pharmacol. 18 801, (1969).

JUNGE, W., and K. KRISCH. Critical Reviews in Food Science and Nutrition. 3 371 (1975).

MAIN, A.R., and P.E. BRAID. Biochem. J. 84 255 (1962).

PAHLICH, E., and K. KRISCH. Z. Physiol. Chem. 350 173, (1969).

REINER, E. Bull Wld. Hlth. Org. 44 109 (1971).