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INHIBITION OF PHENACETIN- AND ACETANILIDE-INDUCED METHEMOGLOBINEMIA IN THE RAT BY THE CARBOXYLESTERASE INHIBITOR *BIS*-[*p*-NITROPHENYL] PHOSPHATE*†

E. HEYMANN and K. KRISCH‡

Physiologisch-Chemisches Institut der Justus-Liebig-Universität
6300 Giessen, Deutschland

H. BÜCH and W. BUZELLO

Institut für Pharmakologie und Toxikologie der Universität des
Saarlandes 6650 Homburg/Saar, Deutschland

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Abstract—The formation of methemoglobin caused by phenacetin and acetanilide in rats is inhibited by simultaneous treatment with *Bis*-[*p*-nitrophenyl] phosphate (BNPP), a new carboxylesterase inhibitor, while the methemoglobinemia after *p*-phenetidine is not influenced by BNPP.

The duration of BNPP action on phenacetin-induced methemoglobinemia and the spontaneous return of liver esterase activity after a single dose of BNPP have been studied.

The enzymatic hydrolysis of the amide bond of phenacetin by rat liver, *in vivo* and *in vitro*, is inhibited considerably less by BNPP than the hydrolysis of acetanilide, monoethylglycine-2,6-xylidide and methyl butyrate, and another amidase may be involved in the deacetylation of phenacetin by rat liver.

IT IS WELL known that free aromatic amines are potent methemoglobin forming agents *in vivo*. They are converted by microsomal *N*-hydroxylation to the corresponding phenylhydroxylamine and nitrosobenzene derivatives (see review by Kiese[1]). Thus *p*-phenetidine, the deacetylation product of phenacetin, leads via its *N*-oxidized metabolites *p*-ethoxyphenylhydroxylamine and *p*-ethoxynitrosobenzene to a pronounced methemoglobinemia.[2–4]

It has been shown by Krisch and coworkers[5–7] that highly purified carboxylesterases (EC 3.1.1.1) from pig liver and kidney not only hydrolyse various carboxylic esters *in vitro* but also certain aromatic amides such as acetanilide and phenacetin. If microsomal esterases are also involved in the cleavage of the amide bond *in vivo* it should be possible to inhibit the methemoglobinemia caused by phenacetin and acetanilide in the rat by appropriate carboxylesterase inhibitors. An esterase inhibitor which in contrast to the organophosphate anticholinesterase compounds such as

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‡Present address: Institut für Physiologisch Chemie der Universität, 23 Kiel, Deutschland.

E 600 or DFP * does not inhibit acetylcholine esterase was described recently by Heymann and Krisch.[8] This compound, *Bis*-[*p*-nitrophenyl] phosphate (BNPP), is rather specific, little toxic (LD_{50} in the mouse 410 mg/kg) and therefore provides a valuable tool for blocking carboxylesterases *in vivo*.

In this paper, experiments on the BNPP inhibition of phenacetin- and acetanilide-induced methemoglobinemia in the rat are reported. In addition, some studies on the spontaneous reactivation of liver carboxylesterase after inhibition by BNPP will be described.

METHODS

1. *Administration of phenacetin*, p-*phenetidine*, p-*nitrophenol*, *acetanilide and BNPP*

In all experiments Wistar rats were used. Their weight, sex, the doses applied and further experimental details are given in the legends of Figs. 1–6 and Tables 1–3. Phenacetin, *p*-phenetidine, *p*-nitrophenol and acetanilide were injected intraperitoneally. Phenacetin or acetanilide were thoroughly mixed in a mortar with 0·9% NaCl containing 0·1% tragacanth to give a 1% suspension. Analogously *p*-nitrophenol was prepared as a 0·1 or 0·25% suspension. *p*-Phenetidine-HCl was dissolved without tragacanth as a 0·25 solution in saline.

BNPP was prepared by condensation of *p*-nitrophenol with phosphorus oxychloride.[9] Unless stated otherwise a 1% solution of BNPP in saline was warmed to a temperature of 40–50° and injected intravenously.

2. *Determination of methemoglobin*

The withdrawal of blood samples and the determination of methemoglobin were carried out as described previously.[10] All methemoglobin levels are expressed in per cent of the total hemoglobin content.

3. *Preparation of rat liver homogenates*

Rat livers were removed immediately after sacrifice and homogenized by a glass-homogenizer with Teflon pestle. For homogenization an ice-cold sucrose medium (0·25 M sucrose, 0·001 M EDTA, 0·01 M Tris–HCl buffer pH 7·4) was used. The volume was adjusted with sucrose medium so that 4 ml of homogenate corresponded to 1 g fresh weight. The protein concentrations of the homogenates were determined by a biuret procedure.[11]

4. *Determinations of enzyme activity*

The hydrolytic activities of the homogenates towards the substrates monoethylglycine-2,6- xylidide, acetanilide,[5] methyl butyrate[12] and phenacetin[6] were determined as described previously.

5. In vivo *return of esterase activity after inhibition by a single dose of BNPP*

Rats received 1·25 ml of a 0·8% BNPP solution in saline per 100 g body weight i.p. The controls were injected with a corresponding volume of saline. The first two groups (controls and BNPP treated animals) were killed 60 min after injection (day 1). On each following day two further groups were sacrificed. The livers of the 5 animals in each group were pooled and homogenized as described above. All activities were expressed as relative activities in percent of those of the control groups.

* Abreviations: BNPP = *Bis*-[*p*-nitrophenyl] phosphate, E 600 = Diethyl-*p*-nitrophenyl phosphate DFP = Diisopropyl fluorophosphate, EDTA = Ethylenediaminetetraacetic acid-Na_2.

RESULTS

1. *Methemoglobin formation after simultaneous administration of phenacetin and BNPP*

After a dose of 200 mg (= 1·12 m-moles)/kg phenacetin i.p. the methemoglobin levels in rat blood increase rapidly and reach a maximum of 23 per cent after 2 hr (Fig. 1). In rats treated simultaneously with 50 mg (0·147 m-moles)/kg BNPP i.v. the

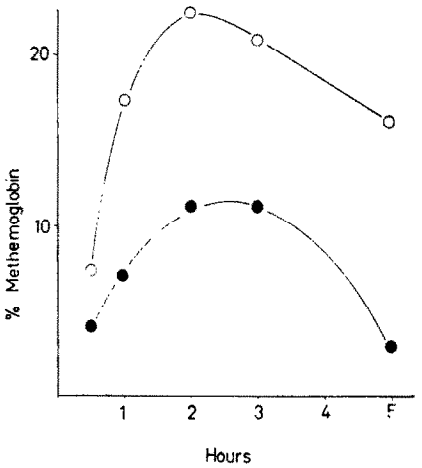

FIG. 1. Methemoglobin concentration (in per cent of the total hemoglobin content) as a function of time after 200 mg/kg phenacetin i.p. (controls ○———○) and after simultaneous administration of the same dose of phenacetin i.p. plus 50 mg/kg BNPP i.v. (●———●). Female Wistar rats, weight about 150 g.

Hours		0·5	1	2	3	5
Controls	*n*	10	12	20	16	4
	σ	± 3	± 4	± 4	± 7	± 7
BNPP	*n*	10	10	10	10	6
	σ	± 1	± 2	± 3	± 3	± 4
P		< 0·005	< 0·0005	< 0·0005	< 0·0005	< 0·0005

methemoglobin concentrations are significantly lowered to about 50 per cent of the values of the untreated control group. This inhibition of methemoglobin formation is not further enhanced by increasing the BNPP dose to 75 mg/kg.

2. *Methemoglobin formation after simultaneous administration of* p-*phenetidine and BNPP*

Following administration of *p*-phenetidine-HCl (50 mg = 0·29 m-moles/kg i.p.) the methemoglobinemia behaves quite differently (Fig. 2). Thirty min after injection of the amine the methemoglobin formation has already reached a level of 45 per cent, i.e. more than twice as much as maximally found after phenacetin. This indicates that *p*-phenetidine is a much more potent methemoglobin forming agent than phenacetin. Subsequently, in spite of the higher initial levels, the methemoglobin declines steeply and after 3 hr it has disappeared almost completely. BNPP, has no significant influence on the *p*-phenetidine-induced methemoglobin formation.

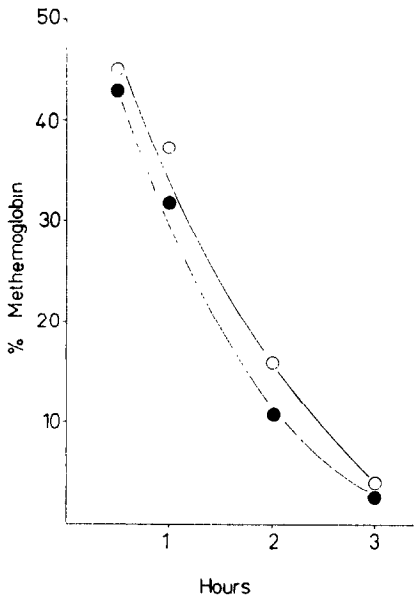

FIG. 2. Methemoglobin concentration (in per cent of the total hemoglobin content) as a function of time after 50 mg/kg *p*-phenetidine i.p. (controls ○———○) and after simultaneous administration of the same dose of *p*-phenetidine i.p. plus 50 mg/kg BNPP i.v. (●———●). Female Wistar rats, weight about 150 g.

Hours		0·5	1	2	3
Controls	*n*	11	11	11	11
	σ	± 4	± 6	± 5	± 3
BNPP	*n*	11	11	11	11
	σ	± 8	± 7	± 5	± 2

3. *Influence of* p-*nitrophenol on the phenacetin-induced methemoglobin formation*

By the reaction of highly purified esterases with BNPP *in vitro* a stoichiometric amount of *p*-nitrophenol is liberated.[8] It was the question, therefore, whether the observed inhibition of phenacetin-induced methemoglobin formation might not be due to BNPP itself but rather to its metabolite *p*-nitrophenol. As can be seen from Table 1 *p*-nitrophenol causes only a slight inhibition of methemoglobinemia which is independent of the administered dose. Further control experiments without phenacetin showed that *p*-nitrophenol (50 mg/kg i.p.) does not produce methemoglobin. It can be excluded, therefore, that a higher inhibitory effect of *p*-nitrophenol might be partially compensated by a simultaneous methemoglobin formation due to this compound itself.

4. *Inhibition of phenacetin-induced methemoglobin formation by subsequent administration of BNPP*

Rats received phenacetin in a dosage of 400 mg/kg i.p. Two hr later one group was injected 50 mg/kg BNPP i.v. (Fig. 3; arrow). The application of the inhibitor within

TABLE 1. INFLUENCE OF *p*-NITROPHENOL ON PHENACETIN-INDUCED METHEMOGLOBIN FORMATION (METHEMOGLOBIN CONCENTRATION IN PER CENT OF THE TOTAL HEMOGLOBIN)

	Hours after application 2	3
200 mg/kg Phenacetin i.p. (= controls)	23 ± 4 $n = 20$	21 ± 7 $n = 16$
200 mg/kg Phenacetin i.p. + 20 mg/kg *p*-Nitrophenol i.p.	19 ± 2 $n = 8$	17 ± 4 $n = 8$
200 mg/kg Phenacetin i.p. + 50 mg/kg *p*-Nitrophenol i.p.	20 ± 4 $n = 6$	17 ± 6 $n = 6$

Female Wistar rats, weight about 150 g. The dose of 20 mg *p*-nitrophenol is equimolar to 50 mg BNPP (assuming that 1 mole *p*-nitrophenol is liberated from the inhibitor.

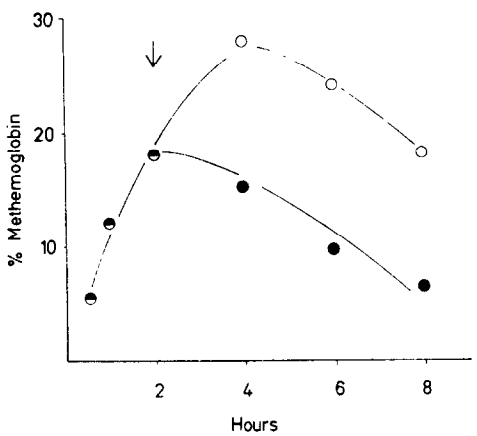

FIG. 3. Inhibition of methemoglobin formation by BNPP after 400 mg/kg phenacetin i.p. 2 hr after administration of phenacetin part of the animals received 50 mg/kg BNPP i.v. (arrow; ●———●). The other animals served as controls (○———○). Male Wistar rats, weight about 250 g.

Hours		0·5	1	2	4	6	8
	n	15	15	15	7	7	7
	σ	± 2	± 4	± 6	± 7	± 9	± 9
	n				8	8	8
	σ				± 6	± 5	± 4
P					< 0·025	< 0·005	< 0·005

the raising part of the curve immediately stops the methemoglobin formation. 2, 4 and 6 hr after administration of BNPP the methemoglobin levels are less than half as compared to the untreated controls.

5. *Duration of action of BNPP*

Rats were pretreated with 50 mg/kg BNPP i.v. Thereafter, in intervals varying from 3 to 144 hr, the animals received 200 mg/kg phenacetin i.p. Two hr after administration of phenacetin the methemoglobin concentration in blood was determined. As is shown in Fig. 4, the inhibitory effect of BNPP is most pronounced

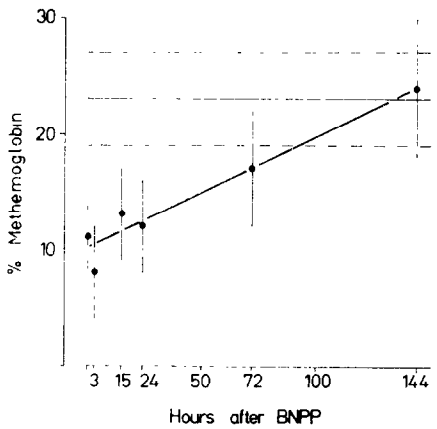

FIG. 4. Duration of BNPP action as measured from the methemoglobin formation after 200 mg/kg phenacetin i.p. All animals were pretreated with 50 mg/kg BNPP i.v. At the times indicated (3–144 hr after administration of the inhibitor) phenacetin was injected i.p. and 2 hr later the methemoglobin concentration was determined. The dotted lines indicate the normal range (± standard deviation) of control animals not pretreated with BNPP ($n = 20$). The number of pretreated animals was 8 for the first point; for all other points n was 10. Female Wistar rats, weight about 150 g.

within the first 3 hr after application of BNPP. Subsequently, the inhibition of methemoglobin formation gradually decreases approximately linearly with time. Seventy-two hr after pretreatment with BNPP there remains still a small but significant inhibition while 6 days (144 hr) later the methemoglobin levels are the same as in the control group again.

6. *Spontaneous return of liver esterase/amidase activity after inhibition by BNPP*

In addition to the experiments on the duration of BNPP action on the phenacetin-induced methemoglobinemia (see Fig. 4) a study was made on the spontaneous return of esterase activity in rat liver. After injection of a single dose of BNPP (100 mg/kg i.p.) one group of animals was sacrificed each day and the hydrolytic activities of liver homogenates towards phenacetin, monoethylglycine-2,6-xylidide and methylbutyrate were determined. The result is shown in Fig. 5. The half-time of the return of esterase activity is about 50-60 hr. The activity curves obtained with the three different substrates run essentially parallel.—Interestingly, on the first day, i.e. 60 min after

BNPP, the residual activity of phenacetin hydrolysis was consistently higher (28 per cent) than the residual hydrolytic activities towards monoethylglycine-2,6-xylidide and methyl butyrate (8 and 10 per cent, respectively).

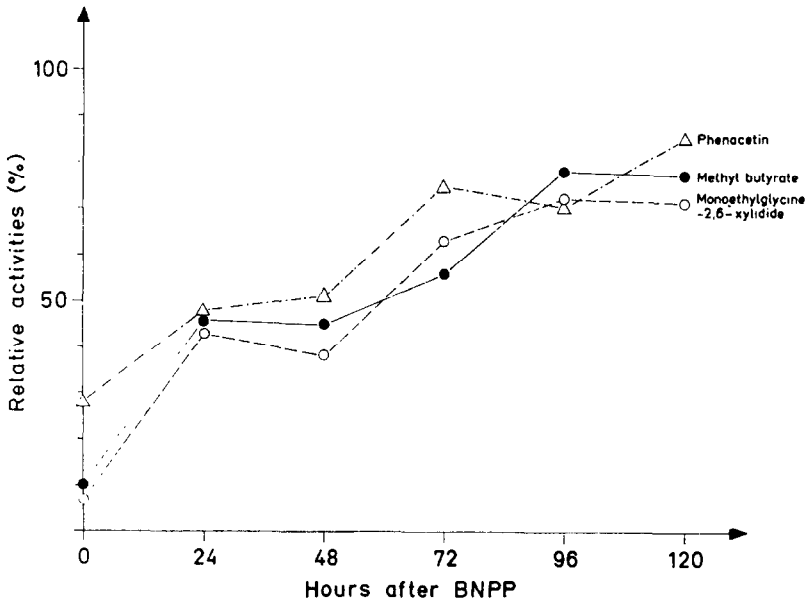

FIG. 5. The rate of return of esterase activity in rat liver after a single dose of 100 mg/kg BNPP i.p. The relative hydrolytic activities of rat liver homogenates were determined using the substrates methyl butyrate (●———●), monoethylglycine-2,6-xylidide (○———○) and phenacetin (△—·—·—△). Male Wistar rats, weight 90–190 g. All activities are expressed as relative activities in per cent of the activities of control groups which had received saline instead of BNPP.

7. Esterase activities of liver homogenates towards phenacetin and acetanilide after administration of BNPP

For further confirmation of this unexpected result rats were treated with BNPP (50 mg/kg i.v.; same conditions as in Fig. 1) and sacrificed 2 hr later, i.e. at the maximum of phenacetin-induced methemoglobinemia. Subsequently, the hydrolytic activities of liver homogenates towards phenacetin and acetanilide were determined. The result is given in Table 2. Under these conditions the enzymatic hydrolysis of

TABLE 2. INHIBITION OF THE HYDROLYTIC ACTIVITIES OF RAT LIVER HOMOGENATES TOWARDS PHENACETIN AND ACETANILIDE 2 hr AFTER 50 mg/kg BNPP i.v.

Substrate	Group	Specific activity*	Relative activity (%)
Phenacetin	controls	1·10 ± 0·11	100
	BNPP	0·70 ± 0·11	64
Acetanilide	controls	1·13 ± 0·32	100
	BNPP	0·048 ± 0·027	4·3

* nmoles substrate hydrolysed × min^{-1} × mg^{-1} protein. Mean and standard deviation; number of animals $n = 5$. Female Wistar rats, weight 145–160 g.

acetanilide was inhibited almost completely, while the deacetylation of phenacetin was inhibited by about 35 per cent only.

8. In vitro *inhibition of the esterase activities in rat liver homogenates by BNPP and E 600*

In addition some inhibition studies were made with rat liver homogenates *in vitro* (Table 3). It was found that the hydrolytic activities towards the substrates

TABLE 3. INHIBITION OF ESTERASE ACTIVITY OF RAT LIVER HOMOGENATES BY E 600 AND BNPP *IN VITRO*

Substrate	Inhibitor	Specific activity*	Relative activity (%)
Phenacetin	—	1·26 ± 0·05	100
	E 600	0·027 ± 0·003	2·1
	BNPP	0·78 ± 0·03	62
Acetanilide	—	1·23 ± 0·10	100
	E 600	0·0	0
	BNPP	0·01 ± 0·009	0·8
Monoethylglycine-2,6-xylidide	—	2·24 ± 0·09	100
	E 600	0·0	0
	BNPP	0·0	0
Methyl butyrate	—	1080 ± 350	100
	E 600	12 ± 10	1·1
	BNPP	22 ± 3	2·0

Inhibitor concentrations: E 600 10^{-4} M; BNPP 10^{-4} M. All samples with BNPP were preincubated 30 min at 37°C with homogenate. Subsequently the enzymatic reaction was started by addition of substrate. During preincubation the activities of the control homogenates decrease by about 10 per cent. If this is taken into account the corrected relative activity towards phenacetin in the presence of BNPP is 68 per cent.

* nmoles substrate hydrolysed × min^{-1} × mg^{-1} protein. Mean and standard deviation; number of animals $n = 3$.

monoethylglycine-2,6-xylidide, acetanilide, phenacetin and methyl butyrate were inhibited almost 100 per cent by E 600 (10^{-4} M). In contrast BNPP (10^{-4} M; after 30 min preincubation with homogenate) caused a more than 95 per cent inhibition of the enzymatic hydrolysis of monoethylglycine-2,6-xylidide, acetanilide and methylbutyrate while under the same conditions the deacetylation of phenacetin was inhibited by about 40 per cent only. Control experiments revealed that the hydrolysis of both acetanilide and phenacetin by highly purified esterase preparations from pig liver and kidney[5, 7] was completely inhibited after 30 min of preincubation with 10^{-4} M BNPP.

9. *Methemoglobin formation after simultaneous administration of acetanilide and BNPP*

Since a considerable portion of the enzymatic hydrolysis of phenacetin by rat liver is resistant against BNPP *in vitro* and *in vivo*, it might be expected that acetanilide would be a better substrate in order to demonstrate an inhibitory effect of BNPP on methemoglobin formation. The influence of the inhibitor on the methemoglobinemia caused by acetanilide is shown in Fig. 6. The acetanilide dose administered (400 mg/kg i.p.) produced about the same methemoglobin levels in the control rats as did 200 mg/kg phenacetin. In contrast to the results with phenacetin (Fig. 1), the

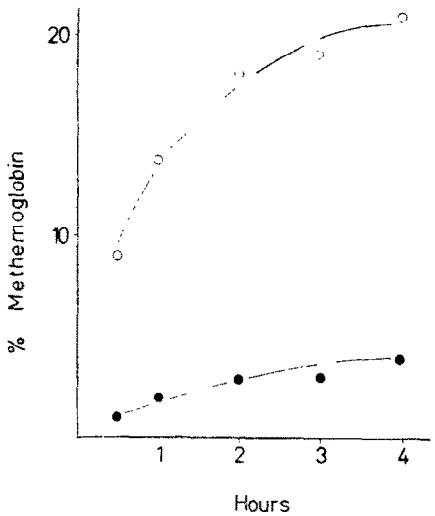

FIG. 6. Methemoglobin concentration as function of time after 400 mg/kg acetanilide i.p. (controls ○———○) and after simultaneous administration of the same dose of acetanilide i.p. plus 50 mg/kg BNPP i.v. (●———●). Female Wistar rats, weight about 150 g.

Hours		0·5	1	2	3	4
Controls	*n*	10	10	10	10	10
	σ	± 2	± 4	± 4	± 5	± 5
BNPP	*n*	10	10	10	10	10
	σ	± 0·5	± 1	± 2	± 2	± 2
P		< 0·0005	< 0·0005	< 0·0005	< 0·0005	< 0·0005

maximum of methemoglobin formation is not yet reached 4 hr after the injection of acetanilide. According to expectation, the inhibitory effect of BNPP is markedly greater in this experiment, the residual methemoglobinemia being only 15–20 per cent as compared to the control group.

DISCUSSION

The results presented here show that the methemoglobinemia caused by phenacetin and acetanilide in rats is significantly inhibited by simultaneous administration of the new carboxylesterase inhibitor BNPP. This indicates that in the rat the amide bond of both phenacetin and acetanilide is hydrolysed *in vivo* by carboxylesterases resembling the highly purified microsomal carboxylesterases from pig liver and kidney,[5, 7*] which are inhibited stoichiometrically by organophosphorus compounds, such as E 600[13] and BNPP.[8] As was shown earlier, these esterases also catalyse the deacetylation of acetanilide[5] and phenacetin[6, 7] *in vitro*. Because of the low turnover numbers, however, the question might be raised, whether these reactions are of significance *in vivo* as well. According to our results this is obviously the case.

The main pathway in the metabolism of phenacetin involves *O*-dealkylation to *N*-acetyl-*p*-aminophenol and subsequent glucuronide and sulfate conjugation,

* So far no carboxylesterase preparations of comparable purity have been isolated from rat liver or kidney.

respectively.[14–16] *N*-Acetyl-*p*-aminophenol is also formed from acetanilide by aromatic hydroxylation. As judged from the excretion of free aromatic amines in urine, only a very small fraction of administered phenacetin and acetanilide is deacetylated *in vivo*.[14, 15] This side-reaction, however, is toxicologically of great importance because the free aromatic amines formed are immediate precursors of the *N*-hydroxylated methemoglobin forming compounds. Obviously, BNPP does not inhibit microsomal *N*-hydroxylation, since it has no effect on the methemoglobinemia caused by *p*-phenetidine.

As shown in Fig. 4, the duration of BNPP action is remarkably long. After a single dose of BNPP it takes about 6 days until the observed inhibition of phenacetin-induced methemoglobin formation has disappeared. In agreement to this are the results of studies on the spontaneous return of liver esterase activity *in vivo* after inhibition by a single dose of BNPP. In this case the time for 50 per cent reactivation was found to be 50–60 hr and it also takes 6–7 days until the activity is fully restored. As we have shown recently, BNPP reacts irreversibly with highly purified pig liver esterase *in vitro*.[8] Under the assumption that this is valid *in vivo* as well, the observed recovery of esterase activity would then be due to a *de novo* synthesis of enzyme protein. The reactivation curves for the three different substrates, monoethylglycine-2,6-xylidide, phenacetin and methyl butyrate, run essentially parallel. This is consistent with the assumption that all three activities might be associated with the same enzyme. With regard to phenacetin, however, an unexpected observation was made. Both *in vivo* and *in vitro* experiments have shown that there remains a BNPP-resistant deacetylation of phenacetin of about 50 per cent. From this it might be concluded that, besides of the carboxylesterase, there occurs an additional enzyme, which is also involved in the hydrolysis of phenacetin by rat liver. This second amidase is similar in so far as it is completely inhibited by E 600 *in vitro* (Table 2), but it does not react with BNPP and it obviously has a higher specificity for phenacetin. This interpretation is in accord with the observation that the BNPP-inhibition of phenacetin-induced methemoglobinemia is about 50 per cent only and that it cannot be enhanced further by increasing the BNPP-dose from 50 to 75 mg/kg.

In contrast to phenacetin, the hydrolysis of acetanilide is almost completely blocked by BNPP *in vivo* and *in vitro*. Consequently, the methemoglobinemia caused by acetanilide is inhibited to a greater extent by simultaneous administration of BNPP (Fig. 6). In this case the methemoglobin levels of the BNPP-treated animals were 15–20 per cent only as compared to the controls. This result indicates that by far the main pathway of acetanilide-induced methemoglobin formation proceeds via a BNPP sensitive enzymatic hydrolysis. The small amount of residual methemoglobin in the BNPP treated animals may possibly be explained by a direct *N*-hydroxylation of the acylated amine, since *N*-acetylphenylhydroxylamine is known to form methemoglobin in erythrocytes *in vitro*.[17, 18]

It is quite possible, that tissues other than liver are also involved in the deacetylation of phenacetin *in vivo*. Thus, in the pig, homogenates of kidney and duodenum have been found to hydrolyse phenacetin as well.[19] The kidney enzyme was isolated recently and identified as a carboxylesterase quite similar to the carboxylesterase from pig liver.[7]

The results presented in this paper are of theoretical interest because they provide an example of an antidotal effect by an enzyme inhibitor.

REFERENCES

1. M. KIESE, *Pharmac. Rev.* **18**, 1091 (1966).
2. H. BAADER, S. GIRGIS, M. KIESE, H. MENZEL und L. SKROBOT, *Archs exp. Path. Pharmak.* **241**, 317 (1961).
3. K. PFLEGER, W. RUMMEL, E. SEIFEN und W. ROTTMANN, *Med. exp.* **6**, 105 (1962).
4. Dissertation W. ROTTMANN, Medical faculty of the university of the Saarland (1966).
5. K. KRISCH, *Biochem. Z.* **337**, 531, 546 (1963).
6. E. BERNHAMMER und K. KRISCH, *Biochem. Pharmac.* **14**, 863 (1965).
7. W. FRANZ und K. KRISCH, *Z. physiol. Chem.* **349**, 575 (1968).
8. E. HEYMANN und K. KRISCH, *Z. physiol. Chem.* **348**, 609 (1967).
9. N. S. CORBY, G. W. KENNER and A. R. TODD, *J. chem. Soc. (London)* **1952**, 1234.
10. H. BÜCH, W. GERHARDS, G. KARACHRISTIANIDIS, K. PFLEGER und W. RUMMEL, *Biochem. Pharmac.* **16**, 1575 (1967).
11. G. BEISENHERTZ, H. J. BOLTZE, Th. BÜCHER, R. CZOK, K. H. GARBADE, E. MEYER-ARENDT und G. PFLEIDERER, *Z. Naturforsch.* **8b**, 555 (1953).
12. W. FRANZ und K. KRISCH, *Z. physiol. Chem.* **349**, 1413 (1968).
13. K. KRISCH, *Biochim. biophys. Acta* (Amsterdam) **122**, 265 (1966).
14. B. B. BRODIE and J. AXELROD, *J. Pharmac. exp. Ther.* **97**, 58 (1949).
15. R. T. WILLIAMS, *Detoxication Mechanisms*. Chapman and Hall, London (1959).
16. H. BÜCH, W. GERHARDS, K. PFLEGER, W. RÜDIGER und W. RUMMEL, *Biochem. Pharmac.* **16**, 1585 (1967).
17. W. HEUBNER, B. WAHLER und C. ZIEGLER, *Z. physiol. Chem.* **295**, 397 (1953).
18. G. HUSTEDT und M. KIESE, *Arch. exp. Path. Pharmak.* **236**, 435 (1959).
19. H. C. BENÖHR, W. FRANZ und K. KRISCH, *Arch. exp. Path. Pharmak.* **255**, 163 (1966).