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Inhibition of sulphation of phenolic substances by the carboxylesterase inhibitor bis-(*p*-nitrophenyl)-phosphate in the rat *in vivo*

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Recently much attention has been paid to the *in vivo* effects of bis-(*p*-nitrophenyl)-phosphate (BNPP), a carboxylesterase inhibitor, which is little toxic and, therefore, well tolerated *in vivo* [1, 2]. It has also been used *in vitro*, for instance for the inhibition of the break-down of aliphatic diesters which induce porphyrin biosynthesis in chicken embryo liver cells [3]. After administration *in vivo*, BNPP is taken up in the liver and is degraded by a phosphodiesterase [2]; during this break-down *p*-nitrophenol is liberated from BNPP. Because *in vitro* *p*-nitrophenol very strongly inhibits sulphation of other substrates [4], presumably competitively, we have studied whether administration of BNPP or its break-down product *p*-nitrophenol might also inhibit sulphation of harmol, a phenolic substrate, in the rat *in vivo*. The results show that BNPP indeed inhibits sulphation of harmol. This inhibition should be taken into consideration in the analysis of results of experiments in which BNPP is intended to be used as inhibitor of carboxylesterase only.

Male Wistar rats (300 g body weight) were used and were anaesthetized by pentobarbital sodium; artificial respiration was applied, the bile duct was cannulated and the kidneys were ligated as described by Mulder and Scholtens [5]. All compounds to be injected were dissolved in aqueous 0.9% (w/v) NaCl, and administered intravenously (in a volume of 3.33 ml/kg in the femoral vein) or intraperitoneally (in a volume of 10 ml/kg) at various time intervals before harmol administration as indicated in Table 1. Harmol (26 μmol/kg) was injected in the femoral vein and bile was collected during 30 min thereafter. In some experiments in rats with intact kidneys urine and bile were collected during 180 min (see Table 1). Urine production was stimulated by infusion of D-mannitol (50 mg/ml in aqueous 0.9% w/v NaCl; 1.9 ml/hr) [6]. The conjugates in bile and urine were separated by t.l.c. and determined fluorometrically as described by Mulder and Hagedoorn [7]. *In vitro* glucuronidation and sulphation of harmol was measured according to the method of Mulder [4] using rat liver microsomes activated with 0.1% (w/v) Triton X-100 as a source of UDP-glucuronyltransferase and a 9000 g supernatant of a 20% (w/v) rat liver homogenate in 0.154 M KCL as a source of sulfotranferase.

At high doses, BNPP decreased the biliary excretion rate of harmol sulfate. This pretreatment enhanced the biliary excretion of harmol glucuronide to 350–400 per cent of control, both after intravenous and intraperitoneal administration of BNPP. The absence of a decrease in harmol sulphate excretion at the low dose of BNPP in 30 min bile of rats with ligated kidneys (a situation previously observed using other selective inhibitors of sulphation [5] may be explained by the slow excretion of this substance in rat bile. When bile and urine were collected during 180 min, a significant decrease in the excretion of harmol sulphate was found and a much higher percentage of harmol was recovered. The major metabolite of BNPP, *p*-nitrophenol, also increased the excretion rate of harmol glucuronide in bile, while it did not affect significantly the excretion of harmol sulfate.

These results might be explained by either inhibition of sulphation or activation of glucuronidation by BNPP or its hydrolysis-product *p*-nitrophenol. Mulder [4] has shown that *in vitro* *p*-nitrophenol inhibits sulphation of harmol and has no effect on glucuronidation. In addition we found that BNPP (10–300 μM) did not affect UDP-glucuronyl-transferase in isolated microsomes. Harmol sulfation was inhibited 18–79 per cent at 10 and 300 μM BNPP respectively. This inhibition was probably due to *p*-nitrophenol, liberated from BNPP by phosphodiesterase present in the 9000 *g* supernatant of rat liver homogenate, because preincubation of BNPP (10 μM) with this homogenate fraction during 25 min resulted in an increase of inhibition of harmolsulphation from 18 to 49 per

Table 1. Excretion of harmol conjugates after pretreatment of rats with bis(*p*-nitrophenyl)-phosphate and *p*-nitrophenol

Pretreatment	n	Dose (μmol/kg)	Time of pretreatment (min)	Harmol sulfate (μmol)	glucuronide (μmol)
A. Bile collected during 30 min. Kidneys ligated.					
Control	9	—	—	0.34 ± 0.10	0.66 ± 0.10
BNPP	2	50 i.v.	5	0.22 – 0.33	2.46 – 2.98
BNPP	4	100 i.v.	5	0.06 ± 0.03*	2.27 ± 0.29*
BNPP	2	300 i.p.	30	0.03 – 0.05	2.06 – 2.36
p-nitrophenol	4	100 i.v.	5	0.27 ± 0.04	1.62 ± 0.35*
B. Bile and urine collected during 180 min. Intact kidneys.					
Control	4	—	—	5.28 ± 0.78	1.46 ± 0.28
BNPP	5	50 i.v.	5	3.44 ± 0.52*	2.55 ± 0.31*

Excretion in bile of harmol sulphate and harmol glucuronide during 30 min; kidneys ligated (A) and 180 min in bile and urine (B) after intravenous injection of harmol (26 μmol/kg; 7.8 μmol/rat).

Bis(*p*-nitrophenyl) phosphate (BNPP) and *p*-nitrophenol were administered at various time intervals ('Time of pretreatment') before harmol administration, in doses and routes as indicated, intravenously or intraperitoneally. The amount of the conjugates excreted is given as means ± S.D.; when only two rats were used, both values have been given.

* Significantly different from Control ($p < 0.005$; Wilcoxon's test).

cent. Moreover, samples containing 300 μM BNPP were a distinct yellow after incubation and centrifugation.

Our conclusion is, that the stimulation of the harmol glucuronidation seen in the present experiments (a secondary effect) is due to an inhibition of sulphation (the primary effect), which leads to an increased substrate availability for glucuronidation. In the case of BNPP, this inhibitory effect may be due to the liberation of *p*-nitrophenol.

An inhibition of sulphation may have played a role in the effect of BNPP on the metabolism of the vasodilator hexobendine (*N,N'*-dimethyl-*N,N'*-bis[3-(3', 4', 5'-trimethoxybenzoyl)propyl]-ethylenediamine), studied by Kolassa *et al.* [8]. This substance is a diester which is hydrolyzed by carboxylesterase activity. The molecule contains six methoxy groups which may be oxidatively demethylated to yield phenolic hydroxyl groups. Demethylated metabolites of hexobendine are excreted for the greater part as glucuronide and sulfate conjugates. Kolassa *et al.* [8] found that after BNPP treatment of rats, more of the compound was excreted in the form of metabolites in bile and less in urine. They explained the increase in the excretion of conjugates, which they have not determined separately, in the bile by an activation of glucuronidation *in vivo*, caused by liberated *p*-nitrophenol. However, our results point to a different mechanism: an inhibition of sulphation of the demethylated hexobendine metabolites, leading to a secondary increase in their glucuronidation. In general, sulphate conjugates are more readily excreted in urine, while their glucuronide counterparts, having a higher molecular weight, are better excreted in bile [9]; a shift from sulphation to glucuronidation will, therefore, often result in an increase of conjugate excretion in the bile.

These results, together with those of Kolassa *et al.* [8] indicate that in the action of the carboxylesterase inhibitor BNPP, also inhibition of sulphation, (and as a consequence an increase of glucuronidation), have to be taken into consideration. Possibly these effects are due to the action of liberated *p*-nitrophenol. They certainly will confuse the analysis of the outcome of experiments in which BNPP was meant to be used to inhibit carboxylesterase only.

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REFERENCES

1. W. Block and R. Arndt, *Biochim. biophys. Acta* **524**, 85 (1978).
2. E. Brandt and E. Heymann, *Biochem. Pharmac.* **27**, 733 (1978).
3. J. K Stephens, I. R. Murphy and G. S. Marks, *Biochem. Pharmac.* **27**, 2431 (1978).
4. G. J. Mulder, *Anal. Biochem.* **64**, 350 (1975.
5. G. J. Mulder and E. Scholtens, *Biochem. J.* **165**, 553 (1977).
6. J. G. Weitering and G. J. Mulder, *Biochem. Pharmac.* in press
7. G. J. Mulder and A. H. Hagedoorn, *Biochem. Pharmac.* **23**, 2101 (1974).
8. N. Kolassa, E. Tuisl and O. Kraupp, *Biochem. Pharmac.* **27**, 2269 (1978).
9. P. Millburn, *Metabolic Conjugation and Metabolic Hydrolysis* (Ed. W. H. Fishman), Vol. 2, p.1. Academic Press, New York (1970).