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ENZYMATIC HYDROLYSIS OF BIS-(4-NITROPHENYL)PHOSPHATE AND BIS-(4-CYANOPHENYL)PHOSPHATE BY RAT TISSUES

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Abstract—The enzymatic hydrolysis of two phosphoric acid diesters, which are known to irreversibly inhibit the unspecific carboxylesterases, was investigated in sixteen organs and tissues of the rat. The highest specific activities were found in the ovaries, whereas the total activities were highest in the liver and the kidney. The complex pH-profiles and the differing activation by Mg^{2+} indicate that several enzymes might be involved in the hydrolysis of these phosphodiesters in all the tissues investigated. In concentrations approximately equimolar to bis(4-nitrophenyl)phosphate (BNPP), the hydrolysis of BNPP is inhibited (10–20 per cent) by pyrophosphate, cAMP and xanthene derivatives, and it is activated up to 50 per cent by ascorbic acid.

The aromatic phosphodiester bis-(4-nitrophenyl)phosphate (BNPP) is arousing growing interest because of its specific inhibitory effect on carboxylesterases (E.C. 3.1.1.1) [1]. BNPP has a very low toxicity and can be used *in vivo*. For example, the *in vivo* inhibition of esterase has an influence on the narcotic-induced porphyrin synthesis [2, 3], or reduces the phenacetin-dependent formation of methemoglobin [4]. BNPP has also been used for the discrimination of esterases [5], and for the titration of active sites [6–8]. Lately, we have used BNPP for investigations on the intracellular protein turnover in the rat [9]. The application of BNPP *in vivo*, however, interferes with the action of the so-called phosphodiesterase IV (E.C. 3.1.4., not further specified by the Enzyme Commission) which has been demonstrated in rat liver [10] and in human tissues [11]. The efficiency of BNPP is reduced if a high percentage of it is hydrolyzed by phosphodiesterases before it can phosphorylate the active site of carboxylesterases. By simultaneous application of BNPP and an inhibitor of phosphodiesterase IV it might be possible to suppress this undesirable activity and to increase the degree of phosphorylation of the carboxylesterase. The problem is to find an inhibitor of phosphodiesterase IV with low toxicity.

The action of phosphodiesterases could also be avoided, if another carboxylesterase inhibitor is found which is not, or only very slowly, destroyed by other enzymes.

Therefore, we investigated the inhibition of BNPP-splitting phosphodiesterases by various inhibitors, and, in addition, the substrate specificity of these enzymes towards bis(4-cyanophenyl)phosphate (BCPP), a BNPP-analogous phosphodiester which is also inhibitory for carboxylesterases [12]. In the first instance we had to systematically investigate the distribution of phosphodiesterase IV in the rat tissues, as nothing was known except for a BNPP-hydrolyzing activity with an acid pH-optimum in rat liver [10].

MATERIAL AND METHODS

Tissue preparation. Female rats (150–200 g) of the Wistar–Hannover strain were killed by decapitation. The isolated organs were homogenized in ice-cold 0.25 M sucrose (1 mM EDTA, 20 mM Tris–HCl, pH 7.4) with a glass-homogenizer with teflon-pestle. The lungs, the stomach, the kidneys, the spleen, the heart, the muscles, and the duodenum were minced with a blender prior to the treatment with the glass-homogenizer. In all cases 4 ml of sucrose solution per g of tissue were added.

The cell fractions were prepared by differential centrifugation [13]. The nuclei, the mitochondria, and the microsomes were resuspended in 0.25 M sucrose so that 2 ml of the suspension corresponded to 1 g of fresh tissue. For several experiments the supernatant from centrifugation at 10,000 g_{max} was used.

The protein content was estimated with a micromodification [14] of the biuret procedure.

Estimation of enzyme activity. Only freshly prepared homogenates and fractions or preparations stored at −20° for 2 weeks maximally were assayed for enzyme activity. The volume of the enzyme samples were 10–30 μl (homogenate, supernatant from 10,000 *g*-centrifugation) and 5–10 μl (nuclei, mitochondria, microsomes), corresponding to 2–6 mg and 2.5–5 mg of fresh tissue, respectively. For the determination of the pH-dependence of the enzyme activity the incubation mixtures contained the universal buffer of Theorell [15] instead of Tris–HCl (see below). One unit (U) is the amount of enzyme which produces 1 μmole of 4-nitrophenol or 4-cyanophenol per min at 30°.

Standard test procedure for the BNPP-hydrolyzing activity. The BNPP-hydrolyzing activity of the homogenates and cell fractions is calculated from the amount of 4-nitrophenol ($\epsilon_{405} = 16{,}500\ M^{-1} \cdot cm^{-1}$) formed. 200 μl of 10 mM BNPP in water are mixed with 770 μl of 0.1 M Tris–HCl pH 8.3 containing 4 mM $MgCl_2$ and 4 mM 2-mercaptoethanol, and are made up to a final volume of 1 ml with enzyme

sample and water. After shaking in a water bath at 30° for 40 min the reaction is stopped with 50 µl of 3 M trichloro-acetic acid. Two blanks, one without enzyme, the other without substrate, are treated in the same way. After centrifugation at 12,000 g_{max} for 2 min 0.5 ml of the clear supernatant is mixed with 1 ml of 0.5 M Tris–HCl pH 8.0, and the absorbance at 405 nm is measured. The degradation of BNPP is proportional with the time and the amount of enzyme, (upper limits: 60 min, and amount of enzyme equivalent to 6 mg of fresh liver).

Standard test procedure for the BCPP-hydrolyzing activity. The enzymatic hydrolysis of bis-(4-cyanophenyl)-phosphate (BCPP) was determined with essentially the same procedure, except that 0.1 M Tris–HCl pH 7.0 was used for the incubation, and that 4-cyanophenol was measured at 280 nm in Tris–HCl-buffer pH 8.0. BCPP has an absorbance maximum at 236 nm, whereas 4-cyanophenol has one at 273 nm (pH 8). The absorbance of BCPP at 280 nm is negligible. The molar absorbance of 4-cyanophenol at 280 nm in Tris–HCl pH 8.0 was found to be 14,700 $M^{-1} \cdot cm^{-1}$.

Experiments with inhibitors of phosphodiesterases. Coffeine, L-dopa, and ascorbic acid were purchased from Merck (Darmstadt, Germany), theophylline from Synochem (Hamburg, Germany), 8-chlorotheophylline and theobromine from EGA (Steinheim, Germany), and cAMP from Serva (Heidelberg, Germany). Opipramol (Insidon) was a gift of the firm of K. Thomae (Biberach, Germany). These substances were dissolved in 0.1 M Tris–HCl pH 8.3 and added to the standard assay mixture to give final concentrations between 0.05–5.0 mM. However, prior to the addition of BNPP all samples, including the inhibitor-containing blanks and the blanks without inhibitors, were pre-incubated at 30° for 10 min. None of the compounds mentioned above influenced the non-enzymic substrate hydrolysis.

RESULTS

Distribution of BNPP-splitting activity. We tested sixteen tissues of the rat for their BNPP-hydrolyzing activity at pH 8.3. The result is shown in Table 1.

Table 1. Hydrolyzing activity for BNPP (2 mM) in various tissues at pH 8.3. The enzyme activities (mean values of eight rats) are given with their S.E.

Homogenate	mU per g of tissue		mU per mg of protein		Mean weight of the organ	mU per organ
Ovaries	660	± 72	7.60	± 1.50	0.1	66
Duodenum	397	± 24	4.48	± 0.48	0.4	159
Kidneys	372	± 22	1.38	± 0.08	1.3	484
Spleen	292	± 22	1.28	± 0.15	0.5	147
Liver	220	± 22	0.71	± 0.07	5.7	1254
Brain	159	± 14	0.75	±0.10	1.8	286
Lungs	101	± 17	0.94	± 0.18	0.9	91
Stomach	67	± 7	0.72	± 0.12	1.1	74
Heart	62	± 4	0.42	± 0.10	0.6	37
Suprarenal gland	94	± 14			0.1	9
Pancreas	59	± 19			0.4	24
Thymus	89	± 20			0.3	27

Besides those organs mentioned in the Table we also tested the *M* psoas, the colon, the marrow, and the blood. In all these cases the activity was almost zero. The highest activities were measured in the ovaries, the duodenum, the kidneys, the spleen and the liver. The specific activity in the ovaries is especially high. However, on the basis of the weight of the fresh organs the activity of the ovaries only amounts to less than 3 per cent of the total activity in the rat. In this respect, the liver and the kidneys contribute most to the total activity. About 50 per cent of the BNPP-splitting activity is localized in the liver.

The subcellular fractions obtained by differential centrifugation from homogenates of liver, spleen, duodenum, and brain show an ubiquitous distribution of the activities at pH 8.3. In the kidneys, however, both the total activity and the specific activity (11.5 mU/g) are highest in the microsomes.

Influence of pH, substrate concentration and metal ions on the hydrolysis of BNPP. Although we were mainly interested in the action of phosphodiesterase IV at physiological pH-values, a report on lysosomal BNPP-splitting activity [10] induced us to measure pH-profiles. All the tissues investigated have a pH-optimum at pH 8 in common (Figs 1a, b). Except for spleen and duodenum, all the organs have a second optimum around pH 5. Moreover, most profiles show a shoulder about pH 6.

The doubling of the BNPP-concentration from 1 mM to 2 mM considerably increases the enzyme activity at pH 8.3, with additional activities ranging from 22 per cent (brain homogenate) to 71 per cent (duodenum homogenate; kidney 57 percent, liver 45 percent, spleen 44 per cent, ovaries 41 per cent). These data correspond to apparent K_m-values in the 1 mM-range.

It is known that the activity of phosphodiesterases often depends on Mg^{2+} or Ca^{2+} [16], therefore we investigated the influence of these ions on the hydrolysis of BNPP. In rat liver homogenate varying concentrations of both $CaCl_2$ and $MgCl_2$ enhance the enzyme activity at pH 8.3, but the effect of Mg^{2+} is more pronounced (opitimal stimulation with 4 mM $MgCl_2$). However, 4 mM Mg^{2+} clearly has no effect on phosphodiesterase IV at acid pH-values (Fig. 2a, b).

The influence of SH-reagents on the stability of the enzyme. The organophosphate hydrolyzing activity is not very stable above 0°. During 24 hr at 4° homogenates usually lose 20 per cent of their activity. However, at −20° the activity was stable for at least 2 weeks. After freezing and thawing three times, the activity of liver homogenate decreased 18 per cent (kidneys 7 per cent, of ovaries 32 per cent).

Some attempts to stabilize the phosphodiesterase IV-activity at pH 8.3 were of varying success. Glutathione at 0.01–4 mM had no effect. Cysteine was slightly inhibitory (up to 27 per cent inhibition of liver homogenate at 12 mM cysteine). Only 2-mercaptoethanol (from 2 to 12 mM) slightly enhanced the phosphodiesterase activity in liver and kidney homogenates. Maximal increase (15 per cent) was found with 4 mM 2-mercaptoethanol. This concentration also stabilizes homogenates stored at 4° up to 24 hr. 4-chloromercuribenzoate, on the other hand, causes only partial inhibition of phosphodiesterase IV. At

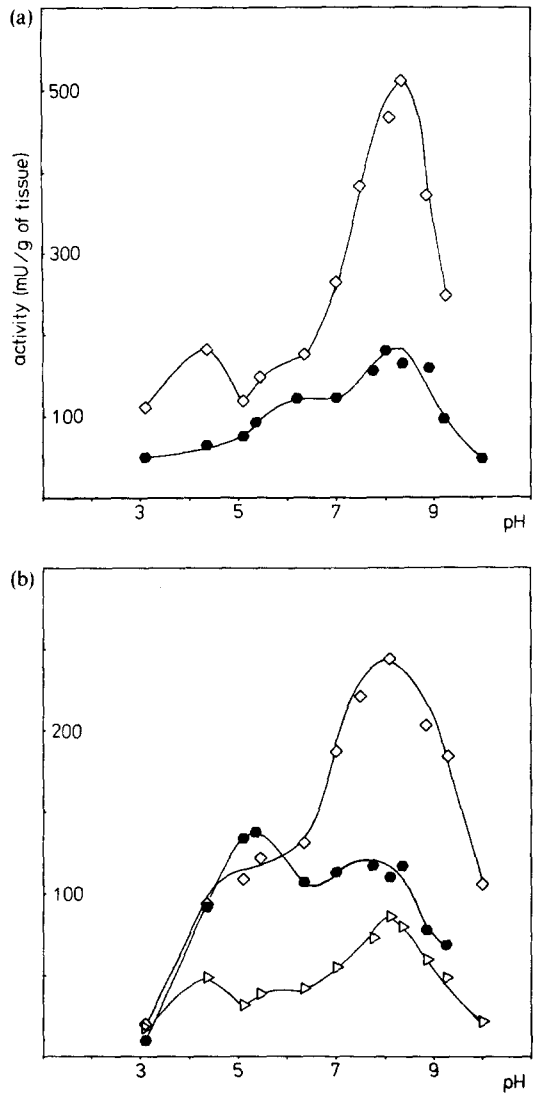

Fig. 1. Influence of pH on the hydrolysis of 2 mM BNPP in various organs (1a) Homogenates of (◇) ovaries, and (●) spleen. (1b) Homogenates of (●) liver, (◇) kidneys, and (△) brain.

all inhibitor concentrations from 0.1 to 1 mM the activity of liver homogenate is reduced to 67 per cent at pH 8.3, and to 54 per cent at pH 7.0. These results suggest that only some of the multiple forms of phosphodiesterase IV have essential SH-groups, others do not.

The influence of known inhibitors of phosphodiesterases. Table 2 gives a survey on the effect of some compounds with low toxicity, which are known to inhibit various phosphodiesterases [16–18], on phosphodiesterase IV in several organs, especially in liver and kidney. Of the xanthene-derivatives only theophylline and coffeine are slightly inhibitory. However, the concentration, necessary for this effect, is not available in experiments *in vivo*. Moreover, the tissue with the highest total activity, the liver, is inhibited only 10 per cent with 5 mM theophylline. The effect of coffeine is similar (Table 2), whereas 8-chlorotheophylline (up to 7 mM) and theobromine (up to 2 mM) are not inhibitory. Of other compounds reported to influence phosphodiesterase activity [16], L-dopa (0.5 mM) and opipramol (0.1 mM) have no influence on phosphodiesterase IV at pH 8.3, ascorbic acid in contrast, activates considerably (Table 2). Pyrophosphate and cAMP show a small but significant inhibition if they are used in a concentration, equimolar to that of BNPP.

Enzymatic hydrolysis of BCPP. In a set of experiments we compared the enzymatic hydrolysis of BCPP, another good inhibitor of carboxylesterase [12], with that of BNPP. The pH-profiles of BCPP (Fig. 3), obtained with homogenates from several organs, are similar to the corresponding curves with BNPP (Figs 1a, b). In both cases, the acid optimum is predominant only in the liver. In Table 3 the standard procedures for the hydrolysis of BCPP (pH 7.0) and of BNPP (pH 8.3) are compared. Both series were measured with identical homogenates. Though the results are very similar, it

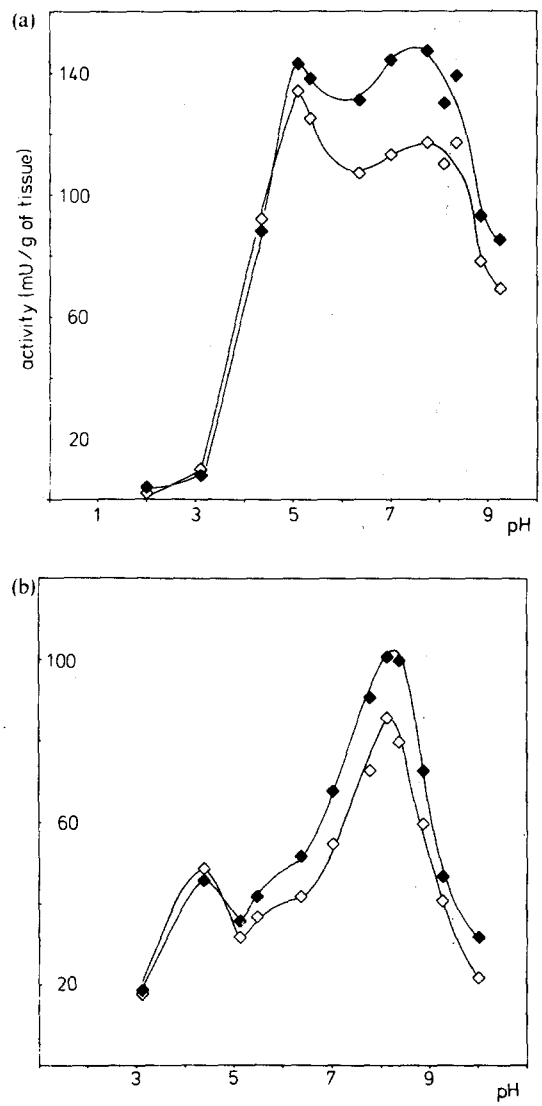

Fig. 2. Influence of Mg^{2+} on the hydrolysis of 2 mM BNPP at differing pH-values. Assay with (◆) or without (◇) 4 mM $MgCl_2$. (2a) Homogenate of liver. (2b) Homogenate of brain.

Table 2. The influence of phosphodiesterase-inhibitors on the hydrolysis of 2 mM BNPP at pH 8.3. Mean values from four to six tests with 10,000 g_{max} supernatants from tissue homogenates are given with their S.E.

Inhibitor	Final conc. mM	Tissue	Remaining activity, %	
Theophylline	1	Liver	95.7	± 3.6
	5	Liver	90.2	± 6.1
	1	Kidney	87.6	± 2.3
	5	Kidney	60.9	± 3.4
	5	Spleen	84.2	± 7.7
	5	Ovaries	68.3	± 2.7
Coffeine	5	Liver	81.2	± 8.6
	1	Kidney	99.1	± 1.3
	5	Kidney	90.7	± 4.2
	5	Spleen	75.2	± 9.7
	5	Ovaries	98.1	± 4.6
Ascorbic acid	5	Liver	147.9	± 2.4
	1	Kidney	125.3	± 1.6
	5	Kidney	130.1	± 3.5
	5	Spleen	117.3	± 2.8
	5	Ovaries	106.2	± 2.3
Cyclo-AMP	0.5	Liver	100.0	± 5.1
	1	Liver*	90.4	± 2.6
Inorg. phosphate	10	Liver*	57	
	100	Kidney	43	
Pyrophosphate	1	Liver*	82.5	±2.5

* Concentration of BNPP = 1 mM, test with liver homogenate.

has to be taken into account that the pH-profiles are usually steep in the pH-range from 7 to 9. Therefore, at pH 8.3 the hydrolysis of BCPP in homogenates of ovaries, duodenum, kidneys, and spleen is much faster than that of BNPP (Fig. 3, Table 3). At pH 7.0 both diesters are cleaved with similar velocities in the liver (Fig. 1, Table 3), whereas the other homogenates again hydrolyze BCPP faster.

DISCUSSION

One of the objectives of this investigation was to obtain a survey of the enzymatic hydrolysis of BNPP and another inhibitor of carboxylesterases in various organs of the rat. The highest specific activities of BNPP and BCPP hydrolysis are found in the ovaries (Tables 1,3). The total activity, however, is much higher in the liver and the kidneys.

It is likely that most of a chemical compound injected intraperitoneally, passes throught the liver. Since BNPP is usually injected intraperitoneally, in order to inhibit the unspecific carboxylesterases of the liver [4], it is possible to estimate the *in vivo*-hydrolysis of this compound.

Five g of liver tissue have a BNPP-hydrolyzing activity of about 1 U (Table 1, Fig. 1). Assuming that 1 mg of i.p. injected BNPP passes quantitatively through the liver, and assuming further that the concentration of BNPP in the liver cells is not higher than 0.1 mg/5 ml at any time, it can be estimated that the intact liver maximally splits 0.2 μmole of BNPP/min (temperature, pH, and substrate concentration differ from the conditions of the standard test).

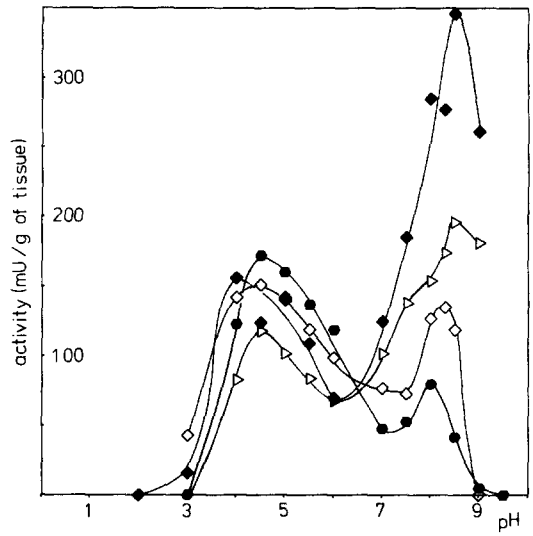

Fig. 3. Influence of pH on the hydrolysis of 2 mM BCPP in various organs. Homogenates of (◆) ovaries, (△) kidneys, (●) liver, and (◇) spleen.

This means, that every 5–10 min one half of the amount of inhibitor present in the cells is cleaved. The complete inhibition of rat liver esterase E1 [8], on the other hand, requires at least 60 min *in vivo* (E. Heymann, unpublished). Therefore, it would be advantageous to use similar inhibitors which are less susceptible to attack by the liver phosphodiesterases. BCPP has no advantage in this respect, however, its toxicity in mice (E. Brandt, unpublished) is still lower than that of BNPP [1].

Though it is not a definitive proof, the pH-profiles and the inhibition experiments with 4-chloromercuribenzoate strongly suggest that more than one enzyme is involved in the hydrolysis of BNPP in a given tissue. The acid pH-optima are probably due to lysosomal enzymes. This activity, which predominates in the liver, has already been described [10]. In all tissues except for liver the main phosphodiesterase IV-activities are found with pH-optima around pH 8. Both in brain and liver phosphodiesterase IV is enhanced by Mg^{2+} only at neutral and alkaline pH-values (Fig. 2). This result corresponds to a similar observation in human liver [11].

The nature of the physiological substrates of phosphodiesterase IV is not clear. Some of these enzymes

Table 3. Comparison of the enzymatic hydrolysis of 2 mM BCPP at pH 7.0 and 2 mM BNPP at pH 8.3. Both tests were performed with the same homogenates

Homogenate	mU/g of fresh tissue BCPP	BNPP
Ovaries	578	639
Duodenum	417	407
Kidneys	385	383
Spleen	361	330
Liver	200	210
Brain	102	142
Lungs	131	166
Stomach	81	73
Heart	45	77

are reported to hydrolyze nucleoside-(4-nitrophenyl)-phosphodiesters [19, 20], others are able to split pyrophosphate [11]. Mg^{2+} and Ca^{2+}, activators of cAMP-phosphodiesterase [16], also stimulate the alkaline phosphodiesterase IV (Fig. 2). Since pyrophosphate and cAMP slightly inhibit the hydrolysis of BNPP in liver homogenate (Table 2), it is possible that both pyrophosphatases and cAMP-phosphodiesterases contribute to the apparent activity of phosphodiesterase IV. On the other hand, inhibitors of the cAMP-phosphodiesterase, namely opipramol [16] and ascorbic acid [17], are without influence, or even activate the phosphodiesterase IV (Table 2). Xanthene-derivatives, which have been reported to inhibit both cAMP-phosphodiesterases [18] and phosphodiesterase IV [16], show some inhibitory effect in our experiments, but only in concentrations which cannot be applied *in vivo*.

Therefore, considering this evidence and because of the apparent heterogeneity of phosphodiesterase IV it is unlikely that an inhibitor of this enzyme can be found, which is capable of increasing the efficiency of BNPP and related compounds as inhibitors of carboxylesterases *in vivo*.

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