THE ACTION OF PHENYLTHIOCYANATE ON ENZYMES

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1. Introduction

Organic thiocyanates are known for their insecticidal, fungicidal and bactericidal properties [1–3]. In recent years, reports in the literature of their ability to act as anti-tumor and anti-hormonal agents [4,5], have given impetus to the synthesis and pharmacological study of a variety of thiocyanic acid esters. However, the mode of action of these compounds at a molecular level is not well understood. Sexton [6] and Zsolnai [3] have indicated the possibility of the interaction of organic thiocyanates with thiol groups in the organism. During our studies on the substrate specificity of the enzyme nitrilase (3.5.5.1) from *Aspergillus niger*, we observed that phenylthiocyanate, which bears a structural resemblance to the substrate phenylacetonitrile, acted as a noncompetitive inhibitor. Similar inhibition was also observed with another C–N bond hydrolysing enzyme, riboflavin hydrolase (3.5.99.1) [7]. Since both of these enzymes, nitrilase and riboflavin hydrolase, are "thiol" enzymes, sensitive to thiol reagents such as p-hydroxymercuribenzoate, *N*-ethylmaleimide etc., we examined the action of phenylthiocyanate on the activity of several enzymes with a view to determine the nature of its action. In this communication some preliminary observations on the interaction between thiol compounds and phenylthiocyanate as well as its action on some enzymes are reported.

2. Materials and methods

Phenylthiocyanate (PTC) was synthesised according to the procedure of Gattermann and Hausknecht [8] and redistilled under reduced pressure (72–75°/6–7 mm). The purity was confirmed by comparison with the IR and UV spectra reported in the literature [9,10]. The thiol compounds used in these investigations were commercial samples of analytical grade. Freshly prepared solutions of phenylthiocyanate in spectroscopic grade ethyl alcohol were used.

Assay procedures: The following enzymes were assayed according to procedures reported in the literature: glyceraldehyde-3-phosphate dehydrogenase [11] yeast alcohol dehydrogenase [12], aldolase [13], glutamic dehydrogenase [14], glutathione reductase [15], lipase [16], phospholipase-C [17], trypsin [18] using casein as substrate, urease [19], ribonuclease [20], nitrilase [21], indoleacetaldoxime hydrolase [22] and riboflavin hydrolase [7]. Papain was assayed by the method used for trypsin [18] after activation with KCN. Alkaline phosphatase was assayed by measuring the inorganic phosphate liberated from phenyl phosphate. In each case an appropriate amount of the enzyme was preincubated at room temperature (23–25°) for ten minutes, with suitable aliquots (5–10 μl) of PTC solution to obtain the final concentrations indicated in table 1 in the assay medium before the addition of the substrate and the coenzyme. Controls were similarly preincubated with equivalent quantities (5–10 μl) of ethyl alcohol.

All reactions were carried out at room temperature unless otherwise stated.

The source and quality of each enzyme used are indicated in table 1. *A. niger* nitrilase was a 30–45% saturation ammonium sulfate fraction of a crude extract of 48 hr old *A. niger* (Mulder) mats grown on glucose-mineral salt medium.

Studies on the interaction of PTC with the functional groups of amino acids such as arginine, histidine, lysine and cysteine were made in a Unicam

Table 1

Enzyme	Assay pH	Concentration of PTC		
		10^{-3} M	10^{-4} M	10^{-5} M
1. Glyceraldehyde-3-phosphate dehydrogenase (*ex.* Rabbit muscle; Sigma 2 X crys.) (1.2.1.12)	8.4 (phosphate)	88	52	– [a]
2. Alcohol dehydrogenase (*ex.* Yeast; Reanal) (1.1.1.1)	7.0 (phosphate)	64	23	– [a]
3. Aldolase (*ex.* rabbit muscle; NBC. 2 X cryst.) (4.1.2.13)	7.5	29	– [a]	– [a]
4. Papain * (Sigma 2 X cryst.) (3.4.4.10)	7.6 (tris)	30	– [a]	– [a]
5. Urease (*ex.* Jack bean; Sigma grade) (3.5.1.5)	7.8	50	– [a]	– [a]
6. Nitrilase (*ex. A. niger*) (3.5.5.1)	7.0 (phosphate)		100	100
7. Indoleacetaldoxime hydrolase (*ex. Gibberella fujikuroi*) (4.2.1.29)	idem		100	100 **
8. Riboflavin hydrolase (*ex. Crinum longifolium*) (3.5.99.1)	idem		100	70

[a] Not tried

* Papain was activated by preincubation with 10^{-3} M potassium cyanide for 30 min at pH 7.6 and then passed through a column of Sephadex G-25 to remove cyanide. The cyanide-free enzyme was then preincubated with PTC and assayed using casein as substrate.

** At 10^{-7} M concentration of PTC 85% inhibition was observed.

PTC at 10^{-3} M concentration had no effect on the activity of the enzymes listed above at indicated assay pHs. Glutamic dehydrogenase (*ex.* bovine liver, Boehringer, 2 X cryst.), pH 7.6 (phosphate); GSSG reductase (*ex.* yeast, Boehringer, 2 X cryst.), pH 7.6 (phosphate); RNase (*ex.* Bovine pancreas, Sigma cryst.), pH 7.0 (phosphate); alkaline phosphatase (*ex.* chicken intestine, NBC), pH 8.0 (tris); lipase (*ex. Rhizopus delamar,* purified by SE-cellulose chromatography), pH 7.2 (tris); phospholipase-C (*ex. Clostridium welchii,* Sigma; purified by DEAE-cellulose chromatography), pH 7.3 (tris); trypsin (*ex.* bovine pancreas, Sigma, 3 X cryst.), pH 7.6 (tris).

automatic recording spectrophotometer model SP 700 A. The reactions were carried out either in pH 7 or pH 8 phosphate buffer, 0.1 M, and at room temperature (22°).

3. Results and discussion

Table 1 shows that the action of PTC is not that of a non-specific inhibition of all enzymes but is restricted to those that are generally regarded as "thiol" enzymes. The inhibitory action of PTC on the activity of glyceraldehyde-3-phosphate dehydrogenase is of particular interest since the involvement of an –SH group in the enzymic reaction is well recognised [23]. The enzymes nitrilase, indoleacetaldoxime hydrolyase and riboflavin hydrolase are known to be inhibited by thiol reagents to varying degrees [24,25,7] and the action of PTC in all these cases is particularly striking.

In view of all these observations spectroscopic studies on the interaction of PTC with the functional groups of amino acids were carried out at pH 7 and 8. Fig. 1 gives the difference spectra of PTC + cysteine at pH 7 and 8 taken against PTC in buffer. The difference spectra of PTC + histidine and of PTC + arginine taken similarly at pH 8 are also given. A characteristic absorption peak at 265–266 mμ appeared only due to an interaction with cysteine which was a time-dependent reaction. Arginine and histidine (fig. 1) and other amino acids tested similarly showed no such interaction. Other thiols such as β-mercaptoethanol and dithioerythritol also gave such absorption peaks with PTC indicating that the –SH function alone interacts, probably as its anion, since the reaction was faster at pH 8 than at pH 7 (figs. 1–3).

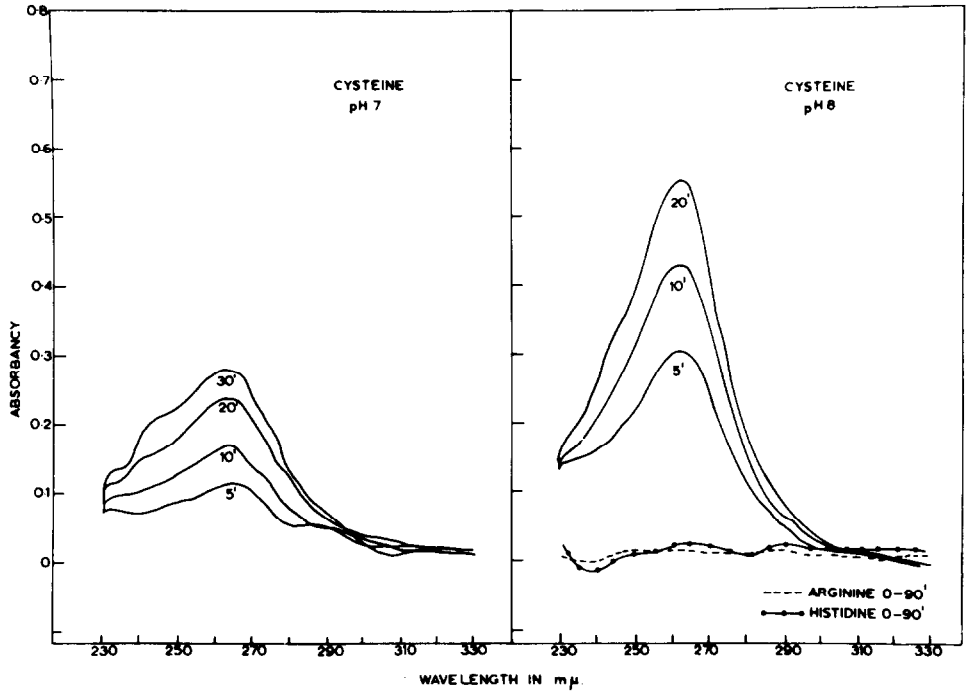

Fig. 1. Difference spectra of the complex formed between PTC and cysteine taken against PTC at pH 7 and pH 8 (phosphate buffer 0.1 M). Final concentrations of PTC and cysteine in the mixture were 10^{-4} M.

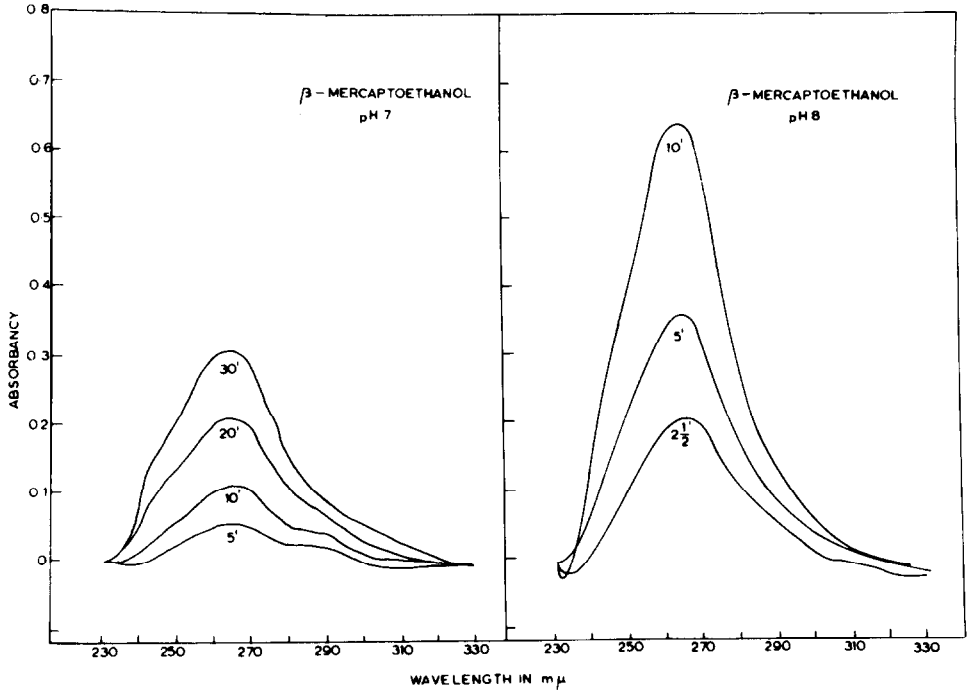

Fig. 2. Difference spectra of the complex formed between PTC and β-mercaptoethanol, taken against PTC at pH 7 and pH 8 (phosphate buffer 0.1 M). Final concentrations of PTC and β-mercaptoethanol were 2×10^{-4} M.

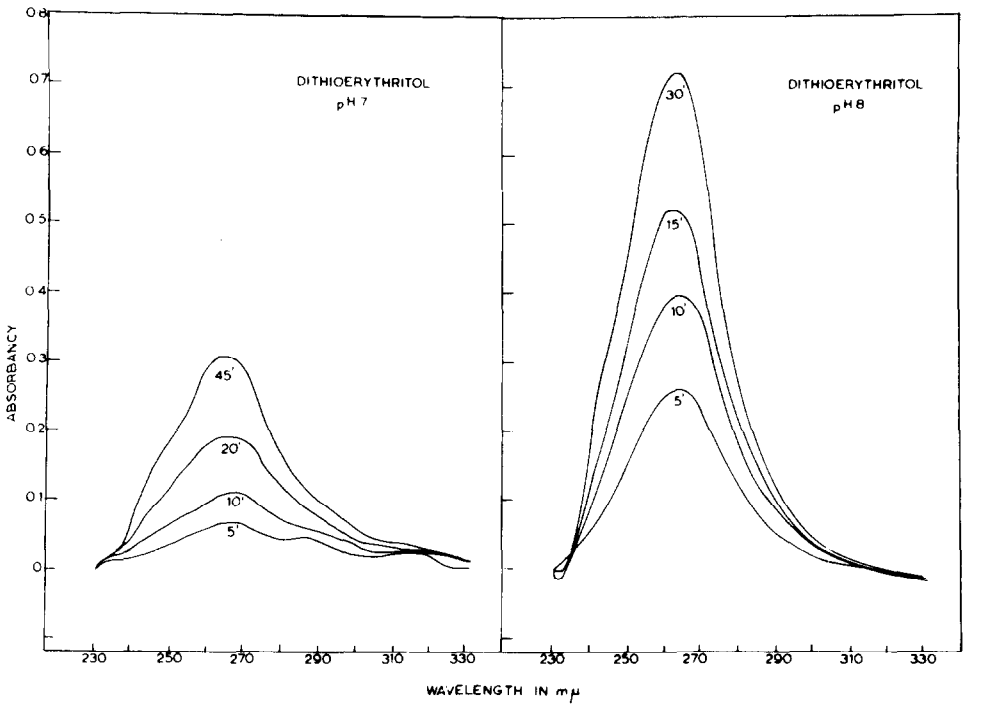

Fig. 3. Difference spectra of the complex formed between PTC and dithioerythritol, taken against PTC at pH 7 and pH 8 (phosphate buffer 0.1 M). Final concentrations of PTC and dithioerythritol were 10^{-4} M.

Hoggarth and Sexton [26], in their studies on tertiary amine catalysed conversion of thiocyanic acid esters to disulfides have postulated an intermediate formation of a thiol which reacts with the organic thiocyanate to yield a disulfide. Similarly the trapping of a mercaptide ion formed during the cyanide cleavage of a disulfide by a thiocyanic acid ester yielding a new disulfide has been described by Hiskey and Harpp [27]. In both these reactions the final steps may be represented as follows:

$$R{-}S^- + R'SCN \rightleftharpoons R{-}S{-}S{-}R' + CN^-$$

In the present investigations it has been clearly demonstrated that among the reactive functional groups of protein amino acids, only the thiol group of cysteine interacts with PTC. That such interactions may occur with the protein–SH groups is demonstrated by the inhibition of several "thiol" enzymes and in particular of glyceraldehyde-3-phosphate dehydrogenase where an –SH group has been definitely shown to participate in the enzymic reaction [23]. Although the enzyme aldolase contains many –SH groups, they are not involved in the enzymic reaction *per se* [28], and hence the enzyme is inhibited only to a small extent by PTC. The lack of inhibition of several enzymes such as trypsin, RNase, phosphatase, lipase etc., which are also not inhibited by other thiol reagents, indicates that PTC does not inactivate these proteins. The very strong inhibitory action of PTC on nitrilase and indoleacetaldoxime hydrolyase may be explained as due to the dual action of PTC's structural similarity to the substrates of these enzymes and its thiol interacting property. These findings suggest that organic thiocyanate structurally resembling the substrates of enzymes may prove useful in elucidating the nature of the active sites of "thiol" enzymes.

Studies on the kinetics of PTC interaction with simple thiols, "thiol" enzymes, the location of PTC on the inhibited enzymes and the reversal of this inhibition are in progress.

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