degree of protection. This data suggests that in the absence of reducing conditions urea causes a partial disruption of the tertiary structure of the enzyme resulting in the exposure and subsequent oxidation of essential -SH groups. When this occurs in a reducing environment, the oxidation of these -SH groups is prevented and the enzyme maintains 40–45% of the native catalytic activity. If, however, the enzyme is either preincubated with NADP or if the exposure to urea is conducted under reducing conditions, the enzyme is only partially inactivated. Finally, if the native enzyme is preincubated with NADP and subsequently treated with 2.0 M urea under reducing conditions, there is no loss of catalytic activity.

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1 H. C. REEVES, B. A. BREHMEYER AND S. J. AJL, *Bacteriol. Proc.*, (1968) 117.
2 T. RAGLAND, T. KAWASAKI AND J. LOWENSTEIN, *J. Bacteriol.*, 91 (1966) 236.
3 H. C. REEVES, B. A. BREHMEYER AND S. J. AJL, *Science*, 162 (1968) 359.
4 D. T. PLUMMER, J. H. WILKINSON AND W. A. WITHYCOMBE, *Biochem. J.*, 89 (1963) 48.
5 I. A. BRODY, *Nature*, 201 (1964) 685.
6 R. RICHTERICH AND A. BURGER, *Helv. Physiol. Acta*, 21 (1963) 59.
7 A. E. H. EMERY, G. E. MOORES AND V. HODSON, *Clin. Chim. Acta*, 19 (1968) 159.
8 S. G. WELSHMAN AND E. C. RIXON, *Clin. Chim. Acta*, 19 (1968) 121.
9 H. C. REEVES AND S. J. AJL, *J. Bacteriol.*, 84 (1962) 186.
10 J. B. MURPHY AND M. W. KIES, *Biochim. Biophys. Acta*, 45 (1960) 382.

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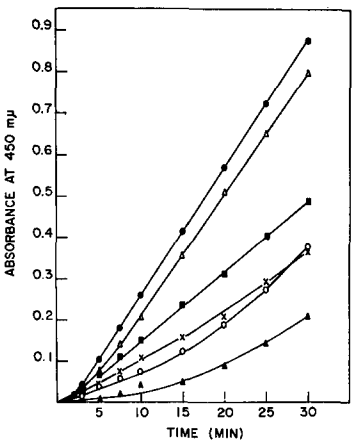
On the effects of cyanide on phenoxazinone synthetase

Phenoxazinone synthetase, an enzyme which is believed to be involved in actinomycin biosynthesis, has been purified from *Streptomyces antibioticus* and studied by KATZ AND WEISSBACH[1]. These workers were unable to demonstrate a cofactor requirement of the enzyme, but they showed that it was affected by various metal ions and chelating agents. An interesting observation was that Cu^{2+} and diethyldithiocarbamate were inhibitory at low levels ($1 \cdot 10^{-4}$ and $1 \cdot 10^{-5}$ M). This suggested to us that phenoxazinone synthetase might be a copper enzyme. The fact that phe-

noxazinone synthetase displays multiple activities[2] (it oxidizes ferrocyanide and catechols in addition to *o*-aminophenols, as in actinomycin chromophore synthesis[1]) is analogous to the multifunctional nature of dopamine β-hydroxylase (EC 1.14.2.1), an enzyme which is known to be a copper protein[3,4].

In this article we report that, when phenoxazinone synthetase was preincubated with cyanide, interesting effects on the kinetics of the reaction catalyzed by the enzyme were obtained. We have also shown that Cu^{2+} or Cu^{+}, but none of several other metal ions tested, is capable of reversing effects brought about by cyanide.

The enzyme was purified essentially as described by KATZ AND WEISSBACH[1] with certain changes and modifications[2]. Preliminary experiments indicated that short-



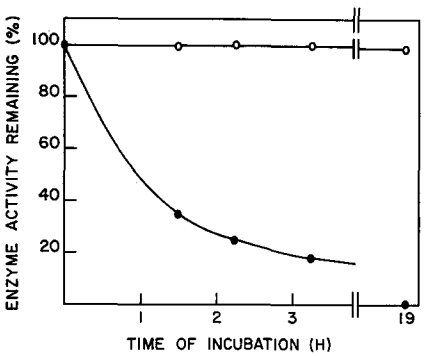


Fig. 1. Kinetics of phenoxazinone synthetase dialyzed *versus* KCN. Phenoxazinone synthetase (6 units and 0.038 mg/ml) was dialyzed against 100 vol. of 0.01 M KCN–0.05 M potassium phosphate, pH 6.5, at 4°. At the times indicated, samples were withdrawn and assayed for enzymatic activity[1] at a final KCN concentration of $1 \cdot 10^{-4}$ M. ●—●, untreated enzyme or any cyanide-treated enzyme samples *plus* $1 \cdot 10^{-5}$ M Cu^{2+} (added as $CuSO_4$); △—△, dialyzed for 21 h; ■—■, dialyzed for 47 h; ×—×, dialyzed for 69 h; ○—○, dialyzed for 93 h; ▲—▲, dialyzed for 168 h. Results were normalized for small volume changes due to dialysis and losses of enzyme activity.

Fig. 2. Sensitization of phenoxazinone synthetase to EDTA by preincubation with KCN. Enzyme (8 units and 0.12 mg/ml) was incubated in the presence of either 0.01 M KCN–0.05 M potassium phosphate, pH 6.5, or 0.01 M EDTA–0.05 M potassium phosphate, pH 6.5, at 0°. At the times indicated, samples were removed and assayed in the presence of either $1 \cdot 10^{-4}$ M EDTA or $1 \cdot 10^{-4}$ M KCN. ●—●, preincubation with KCN, EDTA added to assay; ○—○, preincubated with EDTA, KCN added to assay. Results were corrected for the small inhibition due to effect of both EDTA and KCN on the non-preincubated enzyme.

term (overnight) dialysis *versus* EDTA, diethyldithiocarbamate or cyanide had little or no effect on the enzymatic activity. However, as is shown in Fig. 1, exposure for several days to 0.01 M KCN (pH 6.5) produced an extension of the normal lag phase in the enzymatic reaction. This became quite pronounced after 47 h of dialysis. Although it is not shown in Fig. 1, even with the long lag, the reaction actually approached the velocity of the uninhibited reaction. A final cyanide concentration of $1 \cdot 10^{-4}$ M in the assay incubation was needed in order to maintain the kinetics shown in Fig. 1. At lower concentrations of cyanide, the extended lag phase was shortened. These results indicated that the effect of cyanide was reversible. That this was the case was shown by (1) dialysis of the enzyme to remove cyanide and (2) the addition of

Cu^{2+} to the assay solution, as shown in Fig. 1. The reversing effect due to Cu^{2+} cannot be simply ascribed to complexing of most of the cyanide by the metal ion, since cyanide was present in a 9-fold molar excess with respect to Cu^{2+}. Cu^{+} was as effective as Cu^{2+}. A variety of other metal ions was tested but none was found to simulate the effects caused by copper ions. These included Ca^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, Cr^{3+} and Co^{2+}.

When the dialysis against cyanide was conducted at an enzyme concentration 10-fold greater than that used in the experiments just described, no significant effects on the kinetics were observed, even after 100 h of exposure.

Neither cyanide nor EDTA at $1 \cdot 10^{-4}$ M is a potent inhibitor of phenoxazinone synthetase. However, we have found that preincubation of the enzyme with cyanide can lead to sensitization of the enzyme to EDTA. This is illustrated in Fig. 2. Relatively short preincubation with 0.01 M cyanide led to significant losses of phenoxazinone synthetase activity, when the enzyme was assayed in the presence of $1 \cdot 10^{-4}$ M EDTA. These were actual losses of apparent activity and were not caused by lengthening of the lag phase. However, the sensitivity of the enzyme to EDTA could be reversed by dialyzing the enzyme to remove cyanide. In these experiments the effects could be evoked at higher enzyme concentrations as well.

The results described in this communication indicate that cyanide treatment of phenoxazinone synthetase led to the evolution of two apparent forms of the enzyme: one was normal kinetically, but was EDTA sensitive, and the other was abnormal kinetically as well as EDTA sensitive. The effect of copper ions in restoring normal kinetics does not prove that the enzyme is a copper protein, but a specificity for copper in this action would appear to be indicated. Since dialysis against water reverses the effects of cyanide, it is desirable to study the metal content of the enzyme. In addition, physicochemical studies are needed to see if any correlations may be drawn between the results described here and states of aggregation of the enzyme. The enzyme appears to exist in multiple forms under certain conditions[2].

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1 E. KATZ AND H. WEISSBACH, *J. Biol. Chem.*, 237 (1962) 882.
2 E. GOLUB, Doctoral Dissertation, Tufts University, 1969.
3 S. FRIEDMAN AND S. KAUFMAN, *J. Biol. Chem.*, 240 (1965) PC552.
4 S. FRIEDMAN AND S. KAUFMAN, *J. Biol. Chem.*, 240 (1965) 4763.

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