OXIDATION OF HYDROXYLAMINE BY PLANT ENZYME SYSTEMS

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Frear and Burrell (1955) observed that manganese stimulated the disappearance of hydroxylamine in a crude, cell-free system from soya bean leaves to which reduced diphosphopyridine nucleotide (DPNH) had been added, and concluded that the activity was due to a hydroxylamine reductase. Nason, Abraham and Averbach (1954) inferred the existence of a DPNH-dependent hydroxylamine reductase in Neurospora crassa and mentioned that manganese stimulated the production of ammonia by a soya bean leaf preparation when nitrite was used as the substrate. Zucker and Nason (1955), using Neurospora, observed that DPNH oxidation was associated with the disappearance of hydroxylamine and the formation of ammonia. While investigating hydroxylamine metabolism in higher plants, crude and purified leaf extracts of vegetable marrow (Cucurbita pepo) were found to cause rapid enzymic disappearance of hydroxylamine, in a system whose properties differed markedly from those described for hydroxylamine reductase.

Marrow plants were grown in sand culture (Hewitt 1952) with nitrate supplied thrice daily by automatic irrigation. Extracts were prepared by grinding leaves of four week old plants in a chilled mortar with acid washed sand and three weight-volumes of 0.1M phosphate buffer (pH 7) containing 6.6×10^{-5}M cysteine, and centrifuging the strained brei for 15 minutes at 16,000g (-5°C). The supernate was used, or inert protein was removed on Cγ gel and the enzyme partially purified by elution from calcium phosphate gel

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and precipitation by ammonium sulphate (0.35 - 0.65 saturation) to give a ten-fold increase in specific activity. Hydroxylamine was measured using 8-quinolinol (Frear and Burrell 1955), and by Csaky's (1948) method with similar results. Nitrite was determined according to Evans and Nason (1953). DPNH oxidation was measured on a Hilger Uvispek at 340mμ.

TABLE 1

Factors affecting disappearance of NH_2OH in marrow leaf enzyme system

Concentrations (M) in 8.0ml were: NH_2OH, 2.5×10^{-4}M; Pyrophosphate Buffer, pH 8, 3.7×10^{-2}M; $MnCl_2$, 3.7×10^{-5}M; DPNH, 1.87×10^{-4}M. Boiled Pig Heart Extract, 0.1ml. Marrow enzyme, 1.0ml. Incubation: 15 min. at 29.5°C.

Additional Components and conditions:		NH_2OH loss mμM
PREPARATION I*	Aerobic	1064
	Boiled, Aerobic	0
	Anaerobic	10
	Incubated anaerobically 1 min. before admitting air	368
	Incubated anaerobically with 1.2×10^{-4}M H_2O_2 before mixing and admitting air	236
PREPARATION II*	Aerobic	576
	" : 1×10^{-4} Resorcinol	748
	" : 1×10^{-4} p-Cresol	824
	" : 1.2×10^{-4} H_2O_2	344
	" : 1×10^{-4} p-Cresol + 1.2×10^{-4} H_2O_2	1288
	Boiled : 1×10^{-4} p-Cresol + 1.2×10^{-4} H_2O_2	72
PREPARATION III*	Conditions as for Preparations I and II but incubated 5 min. without DPNH and flavins	
	Aerobic: 1×10^{-4}M p-Cresol + 1.2×10^{-4}M H_2O_2	1270
	Anaerobic: 1×10^{-4}M p-Cresol + 1.2×10^{-4}M H_2O_2	1270

*PREPARATION I, $CaPO_4$ gel elulate; PREPARATIONS II and III, $CaPO_4$ gel elution and $(NH_4)_2SO_4$ pptn.

Under anaerobic conditions negligible activity occurred with, or without the addition of DPNH or reduced triphosphopyridine nucleotide and oxidised or reduced flavin mononucleotide or flavin adenine dinucleotide, boiled pig heart extract or reduced benzyl or methyl viologen. The process appeared to be an oxidation, as air or hydrogen peroxide were found to be essential (Table 1).

Incubation of enzyme with reaction mixture for one minute under anaerobic conditions before admission of air consistently resulted in partial inactivation of the enzyme (Table 1). Similar incubation with hydrogen peroxide under anaerobic conditions before mixing with substrate also inactivated the enzyme. When the enzyme was held separately from the hydrogen peroxide under anaerobic conditions an equally rapid disappearance of hydroxylamine took place on mixing irrespective of whether air was admitted or not (Table 1).

The activity of the system is dependent upon manganese, without which only low activity was observed (Figure 1). Extracts from manganese deficient plants had negligible activities compared to those from full nutrient plants, but they were restored by the addition of manganese to the cell-free systems. Monophenols (p-cresol), resorcinol (Table 1) or boiled extract stimulated activity of purified preparations. These properties resembled the manganese-dependent peroxidation systems elucidated by Kenten and Mann (1952, 1953), and reveiwed elsewhere, Hewitt (1957, 1958) and Ray (1958). No correlation was found between DPNH oxidation and hydroxylamine loss. The addition of 1.87 x 10^{-4} M DPNH decreased the activity of the system in air by almost half but not appreciably in the presence of hydrogen peroxide. In the presence of manganese and marrow enzyme, DPNH was oxidised in air and very rapidly so, with hydrogen peroxide.

In further experiments horseradish peroxidase was found to replace the enzymic component of the marrow leaf extract, when boiled leaf extract and manganese were also present (Figure 2). In the absence of boiled leaf extract peroxidase was active with hydrogen peroxide alone, and was further stimulated twofold by the addition of p-cresol and manganese together (Figure 2). The purified marrow enzyme was inhibited with hydrogen peroxide alone. In the peroxidase-boiled extract system the response to manganese was associated with a lag period; and in the peroxidase-hydrogen peroxide-p-cresol system a pink colour was produced. With peroxidase in the presence of manganese, boiled extract replaced the effect of p-cresol and hydrogen peroxide together (Figure 2). The kinetics of the two systems were different.

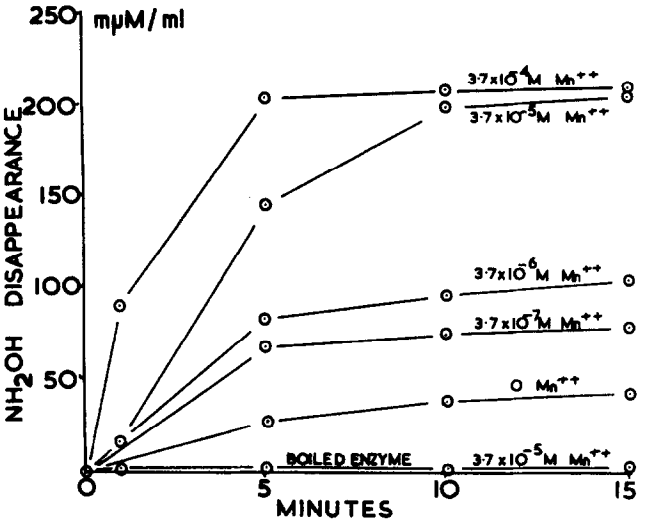

Fig. 1. Effect of Mn concentration on NH_2OH oxidation, in presence of H_2O_2 and p-Cresol, as given in TABLE I for Prep. III.

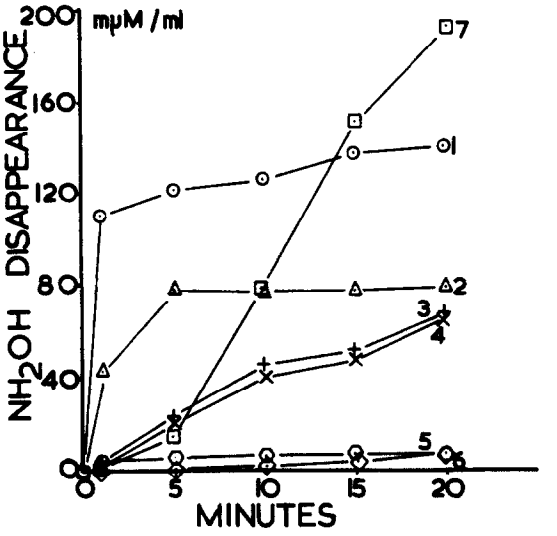

Fig. 2. Effect of Mn, p-Cresol, H_2O_2 and boiled marrow leaf extract on NH_2OH oxidation by horseradish peroxidase; in reaction mixture as for Fig. 1. in phosphate buffer 0.037M, pH 8

1. Mn + p-Cresol + H_2O_2
2. p-Cresol + H_2O_2
3. H_2O_2
4. H_2O_2 + Mn
5. p-Cresol + Mn
6. Boiled Marrow Extract 1.0 ml
7. Boiled Marrow Extract 1.0 ml + Mn

The activity of the marrow enzyme in the presence of p-cresol, manganese and hydrogen peroxide increased sharply from pH 5 to 8. Boiled preparations were inactive over this range and non-enzymic reactions interferred above pH 9. The system was equally active in phosphate and tris buffers, and only slightly less so in citrate and pyrophosphate buffers. Catalase at pH 8 produced no inhibition with the marrow enzyme. In the presence of hydrogen peroxide catechol and pyrogallol caused 25% inhibitions. Unlike the system from wheat leaves (Waygood, Oaks and Maclachlan 1956), maleic hydrazide was unable to replace p-cresol and was inhibitory in the presence of hydrogen peroxide.

Nitrite was produced in small quantities; preliminary manometric studies indicate also gaseous products. Work is in progress to determine their identity. The system described here may well provide a basis for the nitrification of hydroxylamine.

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