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# INTERFERENCE OF PHENOLASE IN THE DETERMINATION OF CYTOCHROME OXIDASE ACTIVITY IN LETTUCE SEEDS

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**Abstract**—Phenolic substances, which become oxidized during the preparation of mitochondria from lettuce seeds, co-precipitate with mitochondria during their isolation. Phenolase present in the mitochondria maintains these phenolics in the oxidized state during the assay of cytochrome oxidase and as a result the activity of the latter enzyme is depressed. The depression is removed by inhibitors of phenolase. This mechanism may also effect cytochrome oxidase activity *in vivo*.

A SLIGHT stimulation of oxygen uptake caused by $5 \times 10^{-3}$ M phenyl thiourea (PTU) was noted during studies on the effect of different inhibitors on the respiration of lettuce seedlings. The effect of this concentration of PTU on cytochrome oxidase activity *in vitro* was therefore studied. Mitochondria were prepared as described by Mayer and Poljakoff-Mayber,[1] and cytochrome oxidase was determined spectrophotometrically by a variation of the method of

TABLE 1. THE EFFECT OF $2 \times 10^{-3}$ M PHENYLTHIOUREA ON CYTOCHROME OXIDASE ACTIVITY

The reaction mixture contained 1·55 ml reduced cytochrome-*c* (0·4 mg/ml) in $KH_2PO_4$ buffer, 0·1 M pH 6·5, 0·05 ml mitochondrial preparation (1·6–1·8 mg protein/ml) and 0·2 ml water or phenylthiourea, $1{\cdot}8 \times 10^{-2}$ M

| Hr of germination | 0 | 24 | 48 | 72 |
|---|---|---|---|---|
| Activity/mg protein (control) | 0·125 | 0·172 | 0·225 | 0·295 |
| Activity/mg protein in presence of phenyl thiourea, $2 \times 10^{-3}$ M | 0·145 | 0·216 | 0·282 | 0·369 |
| Stimulation caused by phenyl-thiourea, % | 15·9 | 25·5 | 25·3 | 25·0 |

Smith[2] described previously.[1] The results are shown in Table 1. A clear stimulation of cytochrome oxidase activity by PTU is evident. Such stimulation might be caused by increased permeability of the mitochondrial membrane, in the presence of PTU. However, pretreatment of the mitochondria with 0·2% deoxycholate for 15 min had no effect on the stimulation caused by PTU, so that this possibility can be excluded.

An attempt was made to determine whether substances closely related to phenylthiourea had a similar effect. Thiourea was found to have a slight effect, causing stimulation of 7–12 per cent at a concentration of $2 \times 10^{-3}$ M, but phenylurea had no effect whatever on cytochrome oxidase activity of isolated mitochondria. Since $2 \times 10^{-3}$ M EDTA was also found

[1] A. M. MAYER and A. POLJAKOFF-MAYBER, *Cell Plant Phys.* **3**, 309 (1962).
[2] L. SMITH, *Methods in Enzymol.* **2**, 732 (1955).

to have no effect on cytochrome oxidase activity the stimulation by PTU was obviously not related to the removal of inhibitory cations.

Lettuce seeds are known to contain up to 1 per cent phenolic substances,[3] as well as an active phenolase[4] which is strongly inhibited by PTU.[5] Some interaction between phenolase and cytochrome oxidase might therefore account for the observed effects.

The effect of inhibitors of phenolase other than PTU on the measurement of cytochrome oxidase activity was therefore studied. Salicylaldoxime at a concentration of $2 \times 10^{-3}$ M was also found to stimulate the cytochrome oxidase activity of the mitochondria. Both in the case of PTU and salicylaldoxime, the stimulation decreased as the concentration was lowered. Experiments with Dieca and ethylxanthate could not be carried out because they interfere directly in the spectrophotometric determination of cytochrome oxidase.

TABLE 2. THE EFFECT OF PHENYLTHIOUREA ON CYTOCHROME OXIDASE ACTIVITY OF MITOCHONDRIA PREPARED IN THE PRESENCE OF ASCORBATE OR PHENYLTHIOUREA

Seeds were germinated for 24 hr and ground in Tris, 0·05 M–sucrose 0·4 M buffer, pH 7·5, which contained either 0·01 M ascorbate or $2 \times 10^{-3}$ M phenylthiourea. The mitochondria isolated from the buffer were washed and then resuspended in buffer without further addition. The reaction mixture as in Table 1

| | Reaction mixture | | % Stimulation caused | |
|---|---|---|---|---|
| | No addition | $+2 \times 10^{-3}$ M PTU | PTU or ascorbate during preparation | PTU in reaction |
| Activity/mg protein (control) | 0·157 | 0·202 | — | 28·7 |
| Activity/mg protein, preparation with 0·01 M ascorbate | 0·190 | 0·203 | 21·0 | 6·8 |
| Activity/mg protein, preparation with $2 \times 10^{-3}$ M PTU | 0·196 | 0·239 | 24·8 | 21·9 |

Since it is well known that the oxidation products of phenols have inhibitory effects on enzyme activity,[6,7] it seemed likely that the stimulation caused by phenyl thiourea was due to its inhibition of phenolase. The effect of PTU on the cytochrome oxidase of mitochondria was therefore studied under conditions where the oxidation of the endogenous phenols was either prevented, or the quinones formed were reduced upon formation. Mitochondria were prepared either in the presence of 0·01 M ascorbate or of $2 \times 10^{-3}$ M phenylthiourea. The results are shown in Table 2. It will be seen that this method of preparation increases cytochrome oxidase activity by about 30 per cent and that PTU had little stimulatory effect on the activity of mitochondria prepared in the presence of ascorbate, and a reduced one on those prepared in the presence of PTU. The effect of phenylthiourea or salicylaldoxime is not due to their forming complexes with quinones. This was determined by studying the absorption spectrum of each of these compounds in the presence and absence of 4-methylorthoquinone. No shift occurred in the absorption spectrum due to the presence of the quinone.

Addition of $2 \times 10^{-3}$ M catechol to the cytochrome oxidase assay system caused an inhibition of activity by 78 per cent. In the presence of PTU or salicylaldoxime this inhibition

[3] W. L. BUTLER, *Nature* **185**, 856 (1960).
[4] A. M. MAYER, *Physiol. Plantarum* **14**, 322 (1961).
[5] A. M. MAYER, *Phytochemistry* **1**, 235 (1962).
[6] J. D. JONES and A. C. HULME, *Nature* **191**, 370 (1961).
[7] E. F. HARTREE, *Advances in Enzymol.* **18**, 1 (1957).

was greatly decreased. The inhibition can therefore be ascribed to the formation of ortho-quinone from catechol by enzymic oxidation. A similar inhibition was caused by the addition of supernatant obtained after separation of mitochondria from a crude extract of the seed. This extract, after dilution 150 times, inhibited cytochrome oxidase activity by 50 per cent, correction being made for non-enzymic oxidation of reduced cytochrome by quinones in the extract.

The data reported here, showing stimulation of cytochrome oxidase activity by PTU, salicylaldoxime and thiourea can be ascribed to their effect on the interaction between cytochrome oxidase and oxidized phenols in the reaction mixture. It appears that some phenolic compounds or quinones precipitate together with the mitochondria during the isolation of the latter. These quinones are reduced by reduced cytochrome-*c* in the assay system. A similar phenomenon has been observed by Kertesz and Zito,[8] while studying the indirect oxidation of reduced cytochrome-*c* by polyphenol oxidase. These authors claim that a considerable error may be introduced by the action of phenolase in the measurement of cytochrome oxidase activity in materials rich in phenolase and its substrates. This error according to them leads to a value of cytochrome oxidase greater than actually present.

The results reported here indicate the reverse phenomenon, in that the presence of phenols and phenolase in the mitochondrial preparation decreases the measured activity of cytochrome oxidase. Quinones present in the mitochondria will be maintained in the oxidized form due to the presence of phenolase, so that an inhibitory level of quinones will be maintained in the reaction mixture. It is these quinones which directly inhibit cytochrome oxidase activity. Inhibition of phenolase by phenylthiourea or salicyladoxime permits the reduction of the quinones to the corresponding phenols and as a result, inhibition no longer occurs and cytochrome oxidase activity rises. This is clearly in contrast to the view of Kertesz and Zito who state that the interaction of phenols and phenolase should cause an increase in the apparent cytochrome oxidase activity, while here a decrease due to such interaction is proposed. However, the view put forward above would account for all the experimental effects described in this paper and possibly also for those of other workers who have found inhibitions due to phenols in mitochondrial preparations.

It is obvious that the observed effects may result from the method of preparing mitochondria and assay of enzyme activity. However, the stimulatory effect of phenylthiourea on oxygen uptake by intact seedlings points to the possibility that a similar situation exists *in vivo*.*

* This work is part of an M.Sc. thesis of E. H.

[8] D. KERTESZ and R. ZITO, *Biochem. Biophys. Acta* **59**, 752 (1962).