

Bhagwat, A. S. and P.V. Sane. Stimulation of macro-molecular synthesis by ascorbate in *Neurospora*.

Although a great deal of information is available in the literature about the nutritional and physiological effects of ascorbate, very little is known about its mechanism of action. Smith (1965 *J. Biol. Chem.* 215: 833) has shown that ascorbate can reduce cytochrome a, a₃ preparations in vitro. It has also been shown by others (Jacobs 1960 *Biochem. Biophys. Res. Commun.* 3: 536) that ascorbate cannot enter the electron transport chain directly but can act as the electron donor through TMPD (tetramethyl-p-phenylene diimine).

In addition to its involvement in oxidative metabolism, there are also reports on the possible involvements of ascorbate in the important physiological activities of plants. Of particular interest is the recent finding of Ehrenberge et al. (1972 *Acta, Chem. Scand.* 20: 1289) of a many fold stimulation of RNA synthesis by ascorbate in plasmolysed *E. coli* cells.

In the light of these studies, it was considered of interest to assess whether the lag in RNA synthesis in *Neurospora* observed by us earlier (Bhagwat and Mahadevan 1970 *Molec. Gen. Genet.* 109: 142) could be influenced by ascorbate. Although the investigations reported here do not provide an unequivocal answer, nevertheless they have revealed some interesting aspects of ascorbate action in *Neurospora*.

N. crassa strain RL3-8A (Rockefeller wild type) was used in all of the experiments. Cultures were grown for about one hour for all manometric studies in 100 ml of 2% sucrose and minimal salts (Vogel 1965 Micro. Gen. Bull. 13: 42) in a shaking water-bath at 30°C. Arcorbote solutions were made freshly before use and the addition of the same to a final concentration of 1 mM did not change the PH of the medium appreciably.

Nuclei from *Neurospora conidio* grown for 8-10 hours were isolated according to the method of Dwivedi et al. (1969 J. Cell Biol. 43: 51) and the *in vitro* RNA synthesis was carried out according to the method of Maitra and Hurwitz (1965 Proc. Nat. Acad. Sci. USA 54: 815). Oxygen consumption, 32 P incorporation into ATP and P:O ratios were determined according to the methods of Nielsen and Lehninger (1955 J. Biol. Chem. 215: 555).

The addition of ascorbic acid to growing cultures of *Neurospora* resulted in increased production of RNA and proteins. On the other hand dehydroascorbate failed to show any such increase in RNA or protein synthesis (Figs. 1,2). Since ascorbate is a reducing agent, we tried to mimic its action by the addition of reduced glutathione. As observed from Fig.1, glutathione did not increase RNA synthesis; on the contrary, it caused a decrease in RNA synthesis. It was also observed that the stimulation

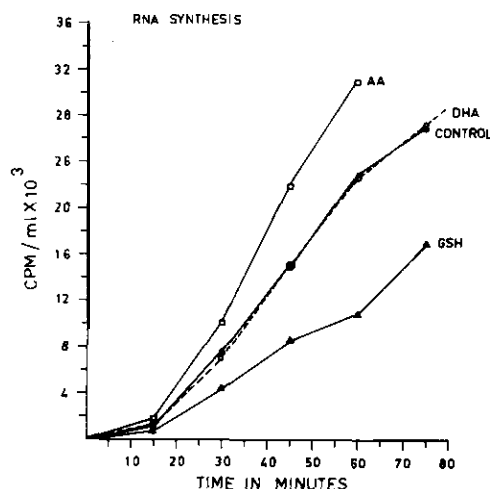


Figure 1. RNA synthesis in conidio grown for different periods of time. The rate of 3 H uridine incorporation into TCA insoluble material was estimated by taking aliquots from cultures growing in the presence and absence of orcorbote.

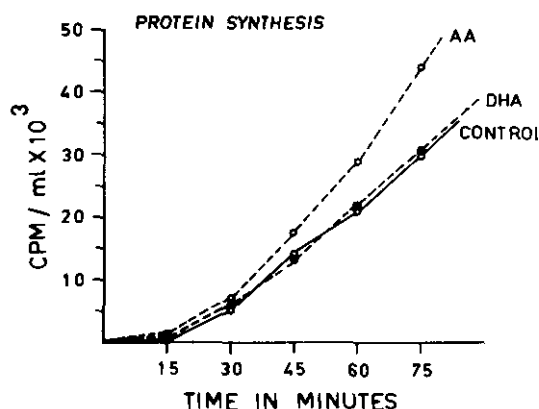


Figure 2. Protein synthesis in conidio grown for different periods of time. The rate of 3 H leucine incorporation into hot TCA precipitating material was estimated by taking aliquots from cultures growing in the presence and absence of ascorbate.

of RNA synthesis was a function of ascorbate concentration with a maximum at 1 mM. In order to investigate the mechanism responsible for stimulation of RNA synthesis, the following possibilities have been considered: a) Increased RNA polymerase activity. b) Removal of an inhibitor (repressor) from the DNA resulting in exposure of DNA for transcription. c) Increased ATP synthesis.

To check the first two possibilities, RNA synthesis has been examined in isolated nuclei in the presence of all components required for an *in vitro* system. The amounts of RNA synthesized in the presence and absence of ascorbate were observed to be similar. This result is at variance with that obtained by Price (1966 Nature 212: 1481), who observed a 40% increase in RNA synthesis in isolated nuclei by ascorbic acid treatment. Our results suggested that ascorbate may not be activating the RNA polymerase per se nor it could be removing an inhibitor bound to DNA (data not presented). If either of these possibilities were true, addition of ascorbate would have been expected to result in stimulation of RNA synthesis. The time lag that is observed in the stimulation of RNA synthesis following addition of ascorbates could be taken as evidence against a direct effect of ascorbic acid, assuming that uptake of ascorbate is not a problem.

The effect of ascorbate may therefore be indirect, being brought about by stimulation of the synthesis of a high energy compound like ATP. This possibility has been investigated by directly estimating the amount of ATP synthesized in whole conidio. The results are shown in Table 1. It is evident that the amount of ATP synthesized in the presence of ascorbate was approximately 30% more than in its absence. Addition of azide, which inhibits mitochondrial electron transport, reduced ATP synthesis more than 60% in the control. The ATP synthesized in the presence of azide and ascorbate is over 25% more than with azide alone. The per cent increase over the respective controls in the presence of ascorbate seems therefore to have been maintained.

Table 1 Effect of ascorbate on the incorporation of 32 P into ATP in conidio of *N. crassa*.

Treatment	CPM/min/0.1 ml
Control	8021
Ascorbate	10500
Azide	3150
Ascorbate + Azide	3918

32 P 10 μ Ci/ml of the medium was added. The reaction was terminated at the end of 5 minutes by the addition of an equal volume of 10% TCA. 32 P incorporation in ATP was estimated as described in materials and methods. Arcorbote conc. 1 mM, Azide conc. 1×10^{-4} M, Conidiol conc. 1×10^7 cells/ml.

Oxygen consumption has also been studied under these conditions in conidia and the results are shown in Tables 2 and 3. The data show that ascorbate addition stimulated oxygen consumption between 30 to 50%. Azide completely stopped oxygen uptake in the control, but in the presence of ascorbate the oxygen consumed was the same as the additional oxygen consumed in the presence of arcorbate over the control. Therefore, the additional oxygen uptake could be through systems other than the normal electron transport chain of mitochondria. It is, however, possible that arcorbate may establish an wide-insensitive pathway of electron transport using a part of the mitochondrial electron transport system.

Table 2. Effect of arcorbate on oxygen uptake by

conidia of *N. crassa*

Treatment	μ l of oxygen consumed per hour
Control	102
Arcorbate	136
Arcorbate + Azide	32
Azide	Nil

The values are averages of 4 separate experiments. Ascorbate conc. 1 mM, Azide conc. 1×10^{-4} M, Conidial conc. 1×10^7 cells/ml.

That the enzyme system responsible for additional oxygen uptake in the presence of ascorbate was localized in the mitochondria was confirmed by studying oxygen uptake by isolated mitochondria from E-hour-old conidia. It was observed that in the presence of arcorbate mitochondria took up about 50% additional oxygen over the control. The data in Table 1 had shown that ATP synthesis by conidia was enhanced in the presence of arcorbate. Table 2 shows the oxygen uptake under identical conditions, as those two experiments were done separately. The oxygen uptake therefore seems to be linked to ATP synthesis. This was confirmed by simultaneously studying oxygen uptake and ATP synthesis in conidia. The results are presented in Table 3. The data show that in conidia in the presence of ascorbate there was approximately a 50% increase in the oxygen uptake, whereas ATP synthesis increased only by 26%. In mitochondria, although the oxygen consumption was stimulated by 50%, the relative stimulation in ATP synthesis was much less, resulting in a lower P:O ratio. This decrease in P:O ratio indicates that ATP synthesis linked to additional oxygen uptake is less efficient.

It is likely that only part of the electron transport chain coupled to phosphorylation may be utilized. Alternatively, the increased ATP synthesis could be from phosphorylation at the substrate level stimulated by arcorbate. These possibilities need to be investigated. In our view the observed effects of arcorbate on stimulation of different metabolic pathways in *Neurospora*, and possibly also in plants and lower organisms, are due to stimulation of ATP synthesis by arcorbate. ■ ■ ■ Biology and Agriculture Division, Bhabha Atomic Research Centre, Bombay 400 085, India.

Table 3. Effect of ascorbate on the P:O ratio in conidia and isolated mitochondria of *N. crassa*.

Treatment	Atoms of O ₂ consumed	Moles of ATP synthesized	P:O ratio
Conidia			
Control	0.66	0.42	0.63
Arcorbate	0.98	0.53	0.52
Mitochondria			
Control	0.28	0.292	1.04
Arcorbate	0.4	0.308	0.75

Conidial conc. 1×10^7 cells/ml, Ascorbate conc. 1 mM, Mitochondrial conc. Equal amounts of mitochondrial suspension was used in each flask, Mitochondrial substrate, 10 mM α -keto glutarate (final conc.), Duration of incubation, 2 hr.