Bhagwat, A. S. and P.V. Sane. Stimulation of macromolecular synthesis by ascorbate in Neurospora.

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

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 arcorbote in plasmolysed E. coli cells.

In the light of these studier, it was considered of interest to assess whether the log in RNA synthesis in Neurospora observed by us earlier (Bhagwat and Mahadevan 1970 Molec. Gen. Genet. 109: 142) could be influenced by arcorbote. 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 RL3-8 A (Rockefeller wild type) war 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 mode freshly before use and the addition of the some 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 ond Hurwitz (1965 Proc. Not. Acad. Sci. USA 54: 815). Oxygen consumption, P^{32} 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 hond 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 glutothione. 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

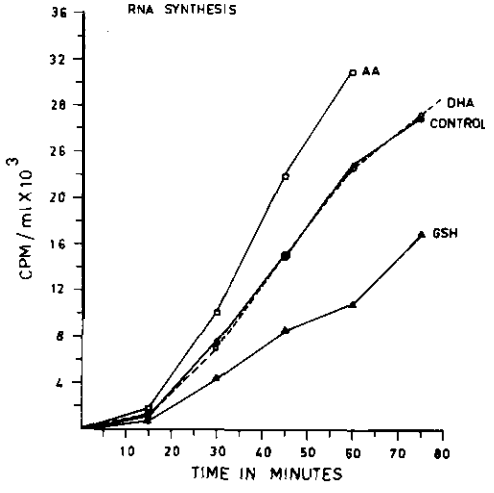

Figure 1. RNA synthesis in conidio grown for different periods of time. The rate of 3H uridine incorporation into TCA insoluble material was estimated by taking aliquots from cultures growing in the presence and absence of orcorbote.

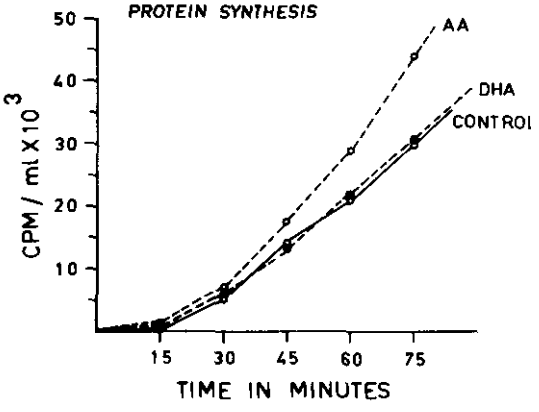

Figure 2. Protein synthesis in conidio grown for different periods of time. The rote of 3H leucine incorporation into hot TCA precipitating material was estimated by taking aliquots from cultures growing in the presence and absence of ascorbate.

of RNA synthesis was a function of ascorbate concentration with a maxium at 1mM. In order to investigate the mechonirm 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 hos 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 thot 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 oscorbote moy not be activating the RNA polymerase per se nor it could be removing an inhibitor bound to DNA (data not presented). If either of there possibilities were true, addition of ascorbate would hove 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 ore shown in Table 1. It is evident thot 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 ond 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 hove 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

32P 10 piCi/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 there conditions in conidio 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 arcorbote over the control. Therefore, the additional oxygen uptake could be through systems other than the normal electron tronrport chain of mitochondrio. It is, however, possible that orcorbate may establish an wide-insensitive pathway of electron transport using a part of the mitochondrial electron transport system.

Table 2. Effect of arcorbote on oxygen uptake by conidia of N crassa

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

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

Table 3. Effect of ascorbate on the P:O ratio in conidio and isolated mitochondrio of N. crassa.

Treatment	Atoms of O2 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. 1x 10^{7} cells/ml, Ascorbate conc. 1 mM, Mitochondriol conc. Equal amounts of mitochondrial suspension was used in each flask, Mitochondriol substrate, 10 mM α-keto glutarate (final conc.), Duration of incubation, 2 hrr.

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 mitochondrio from E-hour-old conidia. It was observed that in the presence of arcorbote mitochondria took up about 50% additional oxygen over the control. The data in Table 1 hod shown that ATP synthesis by conidio was enhanced in the presence of orcorbate. 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 conidio. 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 mitochondrio, 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 moy be utilized. Alternatively, the increased ATP synthesis could be from phosphorylation at the substrate level stimulated by orcorbate. These possibilities need to be investigated. In our view the observed effects of arcorbote on stimulation of different metabolic pathways in Neurospora, and possibly also in plants and lower oraanisms, are due to stimulation of ATP synthesis by orcorbate. - - - Biology and Agriculture Division, Bhabha Atomic Research Centre, Bombay 400 085, India.