The effect of sodium cyanide on inhibiting SOD activity was well recorded by the two procedures.

Although the correlation between the two procedures was very high, NBT reduction procedure always recorded more inhibition than oxygen electrode-based procedure when purified enzyme was used (Table 1). Such observed variations in enzyme activity by the two different procedures are not unusual. In fact, Beyer and Fridovich[4] reviewed a large number of procedures for SOD assay and realized a vast difference in the amount of SOD required for 50% inhibition (0.004–0.73 µg SOD/ml) and pH optima (0.2–10.2) for various assay systems. Our results have shown that the amount of protein required for 50% inhibition by oxygen electrode procedure is about 1.5 times higher than that required for NBT reduction test.

While performing assay with catalases free, extra purified SOD enzyme, it was interesting to note that the addition of 3 U catalase (Sigma Chemicals; cat# C 2001) in the reaction medium showed a further inhibition in the rate of oxygen uptake by oxygraph procedure, but had no effect on NBT reduction (Table 1). Thus, the added catalase catalysed the decomposition of hydrogen peroxide into oxygen and water and the oxygraph recorded this change. Since NBT reacts directly with the superoxide radical and the oxygen molecules *per se* have no effect on it, hence no change was detectable by the NBT reduction method. Hydrogen peroxide in the reaction medium will be generated not only by the action of SOD, but the possibility also exists that self-dismutation of superoxide radicals would generate hydrogen peroxide[6], leading to further release of oxygen by the action of catalases.

When SOD was assayed in crude extracts of tea, pea and barley by the two procedures, inhibition recorded was lower by 58.0, 68.31 and 40.8%, respectively in the NBT reduction method compared to oxygraph-based assay procedures. Our estimations showed very high catalases activity in the crude extracts. This probably explains a higher 'apparent inhibition' recorded by the oxygraph, as discussed earlier. Addition of external catalase in crude extracts had no effect on SOD estimations by the two procedures. Probably, catalases activity in the crude extracts was already present in optimum quantity.

In a separate experiment (Table 1), SOD activity by the two procedures was assayed in crude extract as well as in extra purified enzyme in the presence of externally added hydrogen peroxide (75 nmole) and purified catalase (3 U; Sigma Chemicals; cat# C 2001). The NBT reduction procedure did not show any difference in SOD activity, whereas oxygraph procedure showed further inhibition that was attributed to the externally added hydrogen peroxide. Inhibition thus produced was very much pronounced in case of purified enzyme (139.8% more inhibition) compared to that observed with the crude extract (23–27% more inhibition). While it justifies our hypothesis further, the results with crude extract point out that apart from catalases other hydrogen peroxide scavenging systems, e.g. peroxidases, which would not release any oxygen molecule during catalysis, might also play a role. However, their contribution will depend on the availability of the substrate(s) in the crude extract. Catalatic or peroxidatic activity largely depends upon leaf age, environmental factors and so on. Hence, results obtained with the crude preparations using oxygraph may be misleading.

Some of the known inhibitors of catalases like salicylic acid[7] or aminotriazoles[8] inhibit catalases only partially, while cyanide inhibits catalases as well as copper and zinc containing superoxide dismutase[9]. It was not possible to inhibit either of the enzymes completely to show the role of catalases in overestimation of SOD activity. Therefore, additive rather than the subtractive approach was followed to solve the problem.

While the oxygen electrode-based assay procedure appears to be simple, it does not involve any expensive chemical like NBT and is ideal for coloured preparations, the presence of catalases and other interfering agents, e.g. peroxidases makes it impractical to work with crude preparations. However, the method could be used with highly purified SOD preparations.

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ACKNOWLEDGEMENTS. We thank Prof. S. K. Sinha and Prof. S. D. Ravindranath for providing sodium cyanide and catalase (Sigma Chemicals, USA), respectively. P.K.K. acknowledges UGC, New Delhi for providing Junior Research Fellowship.

SANJAY KUMAR
PRADIP KUMAR KUNDU
PARAMVIR SINGH AHUJA

Biotechnology Division,
CSIR Complex Palampur,
P.B. No. 6,
Palampur 176 061, India

D. Bhatnagar's response:

The determination of superoxide dismutase (SOD) by oxygen electrode (*Curr. Sci.*, 1995, **68**, 960–962) has not been presented for crude preparation and can be used, only with purified enzyme. The crude homogenates cannot be used in this method due to high amounts of peroxidase and catalase and other proteins which should be removed. However, when purified enzyme was used, addition of low concentration of cyanide (0.5 µM) to the reaction mixture inhibit catalase completely without affecting measurement of SOD activity. Low concentration of cyanide (5–50 µM) has been used to inhibit peroxidase and cytochrome oxidase in crude samples while high concentration of cyanide (1–2 mM) has been employed in the differential cyanide inhibition assay to inhibit CuZn SOD and permit the quantitation of both CuZn SOD and Mn SOD (Iqbal, J. and Whitney, P., *Free Rad. Biol. Med.*, 1991, **10**, 69–77). In crude preparations due to the presence of catalase, decomposition of hydrogen peroxide into oxygen will lead to erroneous determination of SOD.

D. BHATNAGAR

School of Biochemistry,
Devi Ahilya Vishwavidyalaya,
Takshila Campus, Khandwa Road,
Indore 452 001, India