GENERAL

DISCUSSION

Nitroso compounds have been proved to be a group of potent chemical mutagen and environmental carcinogen. (67, 70-72). Based on concepts of chemical carcinogenesis, the nitroso compounds have been classified into two groups. One, which requires metabolic activation e.g. nitrosamines and the second, which does not require any metabolic activation e.g. nitrosamides. Important members of the nitrosamines group e.g. dimethylnitrosamine (DMN) and diethylnitrosamine (DENA) and that of nitrosamides group e.g. NTG has been used to study the effect of these compounds on macromolecular synthesis of plant and animal mitochondria.

A general survey of the effect of NTG on mitochondrial protein and ribonucleic acid synthesis from a variety of sources reveal that mitochondrial RNA synthesis is more sensitive to inhibition by NTG rather than mitochondrial protein synthesis (Tables 1, 2 & 3). Secondly, plant mitochondrial protein and RNA synthesis is inhibited to a greater extent in comparison to animal mitochondrial protein and RNA synthesis. In view of the fact that NTG is able to bind with cellular lipopolysaccharides (121), the possibility of binding with mitochondrial membrane component(s) was investigated. Preincubation of isolated plant mitochondria with NTG followed by repeated washings led to partial reversal of the NTG induced inhibition of mitochondrial protein and RNA synthesis (see Figures 2 & 5). Externally added NTG at this stage once again causes similar inhibitions. Thus, so far the effect of NTG on plant mitochondrial protein and RNA synthesis is concerned, it is tempting to conclude that the presence of

NTG is a prerequisite for exerting its inhibitory effect.

Inhibition of plant mitochondrial RNA synthesis by NTG was also investigated at the level of uptake of radioactive precursors. Uptake of radioactive uridine is also inhibited due to the presence of NTG (Figure 4). Thus one of the reason for inhibition of RNA synthesis in isolated plant mitochondria by NTG is due to nonavailability of precursors for RNA synthesis arising out altered permeability. It is also logical to assume that inhibition of RNA synthesis results in an impaired synthesis of mitochondrial proteins to a certain extent. This, however, does not necessarily mean that NTG has no effect at the translational level. Alteration in amino acid acceptor activity of rat liver t-RNA by NTG has been reported previously (125). In addition, modification through methylation of some essential factors in cell-free protein synthesis in rat liver has also been suggested (123).

From earlier experiments in the present investigation it has been concluded that NTG exerts it inhibitory effect on RNA and protein synthesis in isolated mitochondria. It is also likely that interference of RNA synthesis may be due to its binding with mitochondrial DNA. This fact was therefore taken into consideration. It is interesting to note the effect of NTG on plant mitochondrial DNA synthesis is also quite similar to that obtained with plant mitochondrial protein and RNA synthesis (Table 14, Figure 14). Here also, it has been observed that in presence of NTG, there is an inhibition in the uptake of radioactive dGTP (Figure 14). Similar to previous observations

(Chapter I, Section A) this inhibition of plant mitochondrial DNA synthesis is also partially reversible (Figure 15). Therefore, it seems that NTG perhaps forms a loose complex resulting in an altered permeability of the plant mitochondrial membrane. The altered permeability in turn may result in an inhibition of uptake of corresponding radioactive precursors leading to an apparent inhibition of RNA and DNA synthesis in isolated plant mitochondria.

While investigating the mode of action of inhibition of nucleic acid by NTG, three possibilities were taken into account - (a) uptake of nucleic acid precursors into plant mitochondria may be inhibited, (b) mitochondrial RNA polymerase as well as DNA polymerase may itself be inhibited, (c) a combined effect of the above two processes may lead to ultimate inhibition of plant mitochondrial nucleic acid synthesis.

After having arrived at the conclusion that the inhibition of uptake of nucleic acid precursor in presence of NTG is partially reversible, the second possibility was explored. As mentioned before, attempts to isolated the polymerase enzyme from isolated plant mitochondria remained unsuccessful. Considering the universal nature of polymerase enzyme (198), purified polymerases from different sources were therefore used.

DNA synthesis catalyzed by homogeneous E. coli DNA polymerase I and RNA polymerase are inhibited by high concentration of NTG (Tables 17 & 20). Again AMV-DNA polymerase is also completely inhibited. Other homogeneous polymerases e.g. DNA

polymerase, α from acute lymphoblastic leukemic cells and β from human placenta are more or less inhibited (Table 18) to similar extent. Another interesting feature is that the inhibition of E. coli DNA polymerase I and AMV-DNA polymerase activities by NTG is released with increasing concentration of the enzyme respectively (Figures 20 & 21). These release of inhibitions do suggest that NTG possibly inhibits enzyme activities by binding with the enzyme protein itself.

From the present investigation it now becomes clear that NTG inhibits the biosynthesis of mitochondrial nucleic acids at two stages - (i) it inhibits the uptake of radioactive precursors by loosely associating with mitochondria and (ii) it inhibits the RNA and DNA polymerases by binding with the enzyme proteins.

The effects of NTG on macromolecular synthesis have been studied in several strains of E. coli. NTG exhibits a pronounced effect on protein synthesis and a much lesser effect on RNA synthesis. This effect involves the reduction in rate of synthesis of proteins and the production of small amount of non-functional proteins. NTG not only strongly inhibits protein synthesis, but also cause misreading of genetic code (239). NTG can also interact with either the RNA or the proteins in the ribosomes. A direct action of NTG on nucleic acid has been reported (240). The apparent sensitivity of specific enzymes to inactivation by NTG and the evidence that its effect on protein synthesis includes faulty translation leads to the possible explanation of its mutagenic action by interaction with the enzymes related to DNA replication. For example, the interaction

with the DNA polymerase may cause the enzyme to make errors in incorporation of the proper bases, acting as a phenotypically mutagenic DNA polymerase (239). From the studies on the effect of NTG on *in vitro* DNA synthesis catalyzed by *E. coli* DNA polymerase III it has been found, as mentioned earlier, that both the polymerase and associated exonuclease activities are inactivated without any effect on template (169).

Mammalian cells exposed to NTG show an immediate inhibition of DNA, RNA and protein synthesis, as judged by radioactive precursor incorporation (241). DNA synthesis as measured by ^{3}H-thymidine uptake is most sensitive in this respect. Inhibition in this case, however, is not due to impaired uptake or phosphorylation of precursor. Studies at the enzyme level show DNA polymerase to be inhibited by NTG in a dose-dependent manner and that enzyme kinetics favor a mixed mode of enzyme inhibition (241). The nitrosoamides are lipophilic (242) which would favor concentration of NTG in lipid-rich structures, such as cell--membrane and lead to sensitivity of membrane associated events. Inhibition of testicular DNA synthesis by chemical mutagens and carcinogens has been proposed as a simple and effective *in vivo* mammalian screening test (243). Modification of rat liver chromation by NTG and its template activity for RNA synthesis by *E. coli* RNA polymerase after reconstitution have been shown. NTG and its ethyl analog binds significantly to both histone and non-histone proteins. However, the binding of both compounds to DNA was very low and its significance was hard to evaluate (244).

As pointed out earlier, the second group of nitroso compound i.e. the nitrosamines (DMN and DENA) exert its biochemical effects after being converted into an active metabolite. A parallel investigation on macromolecular synthesis in plant mitochondria was therefore carried out subsequently to understand its mode of action. Here also, it was found that there is inhibition of incorporation of radioactive precursors into protein, RNA and DNA of isolated mitochondria (Figures 6 & 8, Tables 15 & 16). It is however important to note, that in contrast to NTG induced inhibition, DMN induced inhibitions of protein, RNA and DNA synthesis is completely reversible (Figures 7, 9 & 18). Again uptake studies also suggest an alteration in the permeability of mitochondrial membrane (Figures 10, 16 & 17) in case of plant mitochondrial RNA and DNA synthesis.

When in vitro DNA synthesis is catalyzed by E. coli DNA polymerase I in presence of either DENA or DMN there is no inhibitory effect. With DMN there is even stimulation (Tables 21 & 23). The stimulation has been further explained as a favorable modulation of "activated" calf-thymus DNA which is used as template in the in vitro DNA synthesis (Tables 22 & 24). Furthermore, it has been shown that mitochondrial extract perhaps does not possess the requisite enzyme to convert the nitrosamines into active metabolite or that even produced, the active metabolite has no inhibitory effect on DNA polymerase I catalyzed in vitro synthesis of DNA (Table 25).

When seeds are germinated in presence of DMN it has been

observed that *in vitro* incorporation of radioactive precursors are stimulated in isolated mitochondria from such seeds, but *in vivo* incorporation is inhibited (see Tables 4 & 5). The stimulation has been attributed to template modulation by nitrosamine while the inhibition has been interpreted as altered permeability of mitochondrial membrane by the carcinogen, inspite of a modulated template. A detail discussion of this data is presented elsewhere (Chapter I, Section A).

If we look at the above inhibition data from the view point of mitochondrial biogenesis, a different line of explanation can be advanced. It is now well known that biogenesis of mitochondria results from a cooperative interaction between nucleo-cytoplasmic as well as mitochondrial genetic apparatuses (10). It is also well known that nitrosamines interfere with cytoplasmic protein synthesis at the level of polysome (127-132). Thus, it may well be a reason to say that inhibition of cytoplasmic protein synthesis leads to an inhibition of mitochondrial protein synthesis.

It is now possible to draw a conclusion on the mode of action of nitrosamines in relation to its inhibition of macromolecular synthesis in plant mitochondria. Following facts have emerged out. Firstly, in contrast to NTG, inhibition of macromolecular synthesis by nitrosamine is completely reversible. Secondly, during mitochondrial RNA and DNA synthesis, uptake of radioactive precursors are drastically inhibited. Thirdly, nitrosamines have no inhibitory effect on purified DNA polymerase.

It is therefore logical to suggest that the observed inhibition of macromolecular synthesis by nitrosamines is only due to inhibition of uptake of radioactive precursors resulting from an altered permeability of the mitochondrial membrane. In other words, nitrosamines act at the level of mitochondrial membrane without having any effect on the polymerase enzyme. Thus the mode of action of nitrosamine (DENA & DMN) differs from that of nitrosamide (NTG) in the following respect. While the former inhibits at the level of mitochondrial membrane, the latter inhibits both at the level of membrane as well as the enzyme.

Cellular DNA polymerases in rat liver are also not inhibited by DENA. If fact sequential changes in DNA polymerase α and β during DENA induced carcinogenesis has been reported (245). It has often been suggested that the high molecular weight DNA polymerase α of eukaryotes plays a role in de novo replication of DNA, while the low molecular weight polymerase β is involved in repair replication (246). Study of DNA polymerase activity in livers of animals during carcinogenesis showed that an increase in polymerase α occurred at the time of increased de novo replication, while there was a gradual increase in polymerase β during the time DENA was present in the diet (245). On the other hand inhibition of mitochondrial DNA synthesis in rat liver has been observed in case of DMN-induced carcinogenesis (126). Inhibition of ^{3}H-thymidine incorporation into DNA of rat esophageal epithelium by DMN has also been reported (247). DMN induced structural damage to DNA has been reported to result from repair of O-6-methylguanine rather than repair of 7-methyl guanine (247,

248). Administration of DMN or DENA to rats have been found to result in decreased sedimentation rates of liver DNA in alkaline sucrose gradients. Time and dose dependencies of both the formation and repair of single strand breaks in rat liver DNA during nitrosamine carcinogenesis has also been reported (249). DNA repair in mutagen injured higher plants have also been reported (250).

In addition to these environmental carcinogen and mutagen, the plant mitochondrial DNA synthesis has been examined with respect to the effects of some known inhibitors of DNA synthesis (Chapter II) e.g. ethidium bromide, pyridoxal phosphate, phosphonoacetic acid and arabinofuranosyl cytosine triphosphate. It is interesting to note that so far in vitro plant mitochondrial DNA synthesis is concerned, only ethidium bromide proved to be a potent inhibitor (see Table 7). This is in confirmation to the earlier reports on the mode of action of this intercalating agent suggesting the involvement of mitochondrial DNA polymerase (181-183). The other three known inhibitors (173-175, 180, 184, 186, 187), however, failed to have any effect on plant mitochondrial DNA synthesis. Source dependent variation in inhibition of DNA synthesis has been well studied in case of phosphonoacetic acid (251) which is a small molecule and inhibits reversibly the multiplication of several herpes and vaccinia viruses. The compound inhibits viral DNA replication by inhibiting the virus-induced DNA polymerase (175, 184). The inhibition is not specific to herpes virus enzyme as originally reported, since a number of eukaryotic DNA polymerase are also inhibited (251).

The biosynthetic capacities of plant mitochondria have also been examined with respect to the effect of DDT, which is a known environmental mutagen and carcinogen. The carcinogenic pesticide DDT differs from carcinogenic nitrosamines in its application potential, and because of a greater chance of exposure to the plant and animal kingdom due to its reckless use over a prolonged period. The results obtained with plant mitochondria suggest that under in vitro condition their biosynthetic capacities is not impaired. However, under in vivo condition an overall stimulation is observed (Chapter I, Section B, Tables 6 & 7). Such stimulation is well in agreement with at least cellular protein synthesis in rat liver (149-154). A close look at the in vivo incorporation data in rat liver and germinating seed with respect to mitochondrial and microsomal protein synthesis reveal that both are inhibited (Tables 8 & 11). This is, however, not the case under in vitro mitochondrial protein synthesis (Chapter IB, Tables 6 & 9). The anomaly can once again be explained by considering the fact that mitochondrial protein synthesis is largely dependent on microsomal protein synthesis. It is important to note that inhibition of microsomal protein synthesis by DDT is in contrary to some of the earlier reports (149-156) where stimulation of hepatic protein synthesis has been observed.

DTT and its metabolite induce chromosomal aberration in both plant and animals (98, 138, 139, 141). In addition to this, secondary effects at the sub-cellular level of organization is also there. Stimulation of hepatic protein synthesis possibly

represent such an effect. Furthermore, DTT has been reported to induce plasmid DNA breakage under *in vitro* condition. Based on this observation attempts are being made to develop a non--mammalian test system for screening potential environmental mutagen and carcinogen (252).

It has been established that fungal and animal mitochondria contain distinct genetic and protein synthesizing systems, which are responsible for the synthesis of probably less than 10% of the total mitochondrial protein. Although the oxidative metabolism of higher plant mitochondria has been clearly defined, the characterisation and function of plant mitochondrial genetic and protein synthesizing systems has, with few exceptions, been neglected. It has been shown, however, that a range of higher plant mitochondrial DNAs have a remarkably constant buoyant density and a contour length, exceeding that of animal mitochondria by a factor of at least six (253, 254). This raises the question as to why plant mitochondria apparently contain so much genetic material than animal mitochondria, to perform the same function. It has been suggested that there has been a conservation during the evolutionary modification of mitochondria, of their ability to synthesize several key proteins of the inner mitochondrial membrane. This suggestion is supported by the fact that increased size of mitochondrial DNA in ascomycete fungi and higher plants is likely to be due to regulatory sequences rather than protein-coding sequence. This in turn again raises the question as to why plants need more control sequences than animals (255).

Moreover, it is intriguing to note that mitochondrial DNA is alkylated three to seven times higher than nuclear DNA in rat liver injected with radioactive MNU or DMN (83, 84). This interesting observation, in fact, was the "primer" to initiate the present investigation on environment carcinogens in relation to mitochondrial functions. The mode of action of nitroso compounds as suggested in the present investigation, put forward yet another receptor site for the mutagenic action of these environmental carcinogen which is different from the nuclear receptor site. This is to say that the sub-cellular DNA target (i.e. mitochondria) is by no means less important than the nuclear DNA target in plant cells, more so because of its larger size and larger regions of regulatory sequences, in comparison to animal mitochondria. Mutations in the regulatory region by these carcinogens is supposed to have a far reaching implication, especially when a cell is destined to undergo extra-chromosomal mutation in plants.
