CHAPTER VII : GENERAL DISCUSSION
The *Salmonella* test was first of all used and recognized as an appropriate carcinogenicity testing system in the study of 300 chemicals most of which were known carcinogens (McCann *et al*., 1975; McCann and Ames, 1976, 1977) and has since been established for the evaluation of the risk of environmental chemicals (Ames, 1984). Although the validity of the test has been questioned in terms of the actual carcinogenic behaviour of the test compound, yet a marked correlation was obtained with several mutagenic compounds tested by the *Salmonella* system (McCann *et al*., 1975; Flessel *et al*., 1987). Our results indicated a higher degree of mutagenicity of the test steroids with GC $\rightarrow$ AT transition mutants, TA102 and TA104 (Levin *et al*., 1982b) compared with those having GC base pairs at the critical site of mutation suggesting that the test steroids preferentially act upon AT base pairs to bring about transition mutation (Table 1, Figs. 1-9 of Chapter III). It is also noteworthy that even in the absence of S9 microsomal fraction, usually all the tester strains responded significantly but the addition of microsomal fraction further enhanced the mutagenic activity of the steroids (Figs. 1, 5, 6, 7 and 8(B) of Chapter III) suggesting that the metabolic products of the test steroids were even more mutagenic.

Comparison of closely related structures allows the identification of a number of features
essential for the mutagenic activity. Comparing steroids I and II which differ only at position 3 of the steroidal nucleus (Table 3 of Chapter II), the acetoxy-derivative was found to be more mutagenic than the chloro-substituted aziridine steroid (Figs. 1 and 2 of Chapter III). Among the halogen-substituted steroids at position 3 (Table 3 of Chapter II), the chlorine-substituted parent was found to be relatively more mutagenic compared with other two parent steroids. This might be due to higher electronegativity of chlorine as compared to bromine and iodine, and thus it could have enhanced the electrophilic potential of sulfur present at the 6th position.

The parent steroids containing an equitorial halogen atom at 3rd position and a reactive thio-moity at the 6th position were further chemically converted into the oxygenated thio-derivatives (steroids IV, V, VII, IX and X, Table 3 of Chapter II). This resulted into further enhancement of mutagenic activity with the increase in number of oxygen atoms in the derivatives (Figs. 4-8 of Chapter III) thereby indicating the probable role of oxygen atoms for the enhanced mutagenicity of the steroids.

Our results are consistent with the idea that in some cases the electronegative group present at the 3rd position of the steroidal nucleus somehow enhances
the electrophilic nature of susceptible moiety present at the 6th position which in turn, upon interaction with some nucleophile in the system generates active oxygen radical species. Although the presence of electrophilic group in the test steroids seems to be desirable in certain cases, it was not probably an essential requirement for these compounds to become mutagenic. In such cases, the bulky groups present at the 3rd and 6th positions might create steric hindrance due to which the overall structure becomes unstable and the groups get cleaved from the parental nucleus. The cleaved groups then probably interact with some reactive species in the system and generate oxygen radical species. Moreover, our results also indicated that the individual moieties/groups were not mutagenic per se (Table 5 of Chapter V). Their association with the steroidal nucleus seems to be essential for their remarkable mutagenicity (Fig.1 and Table 2 of Chapter III).

The tester strain, TA102, has been reported to detect a variety of oxidants and other agents as mutagens (Levin et al., 1982a). A significant amount of inhibition in the mutation frequency of this strain with test steroids was found in the presence of hydroxyl and oxygen radical scavengers (Tables 1-4 of Chapter V). This supports for the production of $\text{H}_2\text{O}_2$ and active oxygen radical species, $\cdot\text{OH}$ and $\text{O}_2^-$ in the test system.
The overall reaction might have been possible according to the following Haber-Weiss reaction (Haber and Weiss, 1934):

\[ \text{H}_2\text{O}_2 + \text{O}_2^\cdot \rightarrow \text{O}_2 + \text{HO}^- + \cdot\text{OH} \]

A possible source of 'OH and \( \cdot \text{O}_2 \) radicals and \( \text{H}_2\text{O}_2 \) in case of the test steroids is summarized in Figs. 1 and 2 (Chapter V).

The higher decrease in genotoxicity with the scavengers observed when these assays were carried out in the presence of liver microsomal fraction compared with those without (Tables 1-4 of Chapter V) further suggests that the Sg dependent reactive species probably differed from those formed chemically.

The results obtained with \( \text{E.coli} \) and \( \text{Salmonella} \) systems suggest that the test steroids bring about the DNA damage and thus the treated cells initiate the SOS-repair with the concomitant induction of mutation. The Ames tester strains carry the pKM101 plasmid which is believed to enhance the error-prone repair process (Levin et al., 1982b; Little et al., 1989). The induction of SOS-response by the test steroids in our case was supported by the high sensitivity of \( \text{recA} \) and \( \text{lexA} \) mutants of \( \text{E.coli} \) towards the test steroids (Fig.1 of Chapter IV). The role of \( \text{recA}^+ \) and
\textit{lexA}^+ \text{ genes in initiating the error-prone repair in } E.\textit{coli} \text{ is well documented (Walker, 1985). A significant amount of induction of prophage in } \lambda\text{-lysogen and requirement of the } \textit{de novo} \text{ protein biosynthesis for this process (Table 1 of Chapter IV) as well as the induction of } \beta\text{-galactosidase in the Mud (Ap, } \textit{lac} \text{) fusion strains (Fig. 2 of Chapter IV) further substantiate our hypothesis. The kinetic studies conducted with steroid-treated Mud lysogens displayed a similar response as with benzo\textquotesingle a\textquotesingle pyrene (Fig. 3 of Chapter IV). This suggests that induction of the operon fusion gene products might occur as a result of a common signal that arises from both, steroid and benzo\textquotesingle a\textquotesingle pyrene treatments. Such type of response was also observed with mitomycin C at low dose (Kenyon and Walker, 1980). The } \textit{in vitro} \text{ studies also favour this postulation since the test steroids at higher doses seem to have induced the interstrand cross-links in the DNA molecule (Chapter VI) which is a well known DNA lesion induced by mitomycin C (Lown, 1977; Remers, 1979).}

The results presented in Chapter VI demonstrate that the test steroid (I) causes a destabilization in the secondary structure of DNA with the formation of ssbs. This is evident by the increased level of single strandedness in duplex DNA as observed by hydroxyapatite chromatography \((Fg1)\) and increased susceptibility to } S_1
nuclease (Table 1) as well as decrease in melting temperature (Fig. 2) at low concentrations of the steroid. Moreover, an appreciable amount of denaturation (local opening) brought about by low dose exposure of steroid is also obvious from the first point of the thermal transition profile (Fig. 2). In addition to these facts, alkaline unwinding assay also suggested the existence of single strand breaks in the treated DNA (Table 3).

An interesting finding of these results is that at higher steroid/DNA bp molar ratio, a greater Tm compared with the control was observed (Fig. 2). This implied a sort of stabilization in the secondary structure of DNA. This result is further strengthened by the experiment shown in Fig. 3 where exposure of DNA to high dose of steroid followed by forced strand separation by boiling the DNA resulted in the incomplete denaturation as compared to the untreated control. A reduced rate of hydrolysis of DNA with S1 nuclease at higher dose of steroid also supports the same idea. As discussed above, these observations can be explained by interstrand cross-linking of DNA at relatively higher concentrations of steroid.

The plausible explanation can be envisaged in view of the modification of pyrimidine bases (Fig. 4) which might disrupt the hydrogen bonding between comple-
In view of the present findings, we propose the following scheme (Fig. 1) for the steroid-induced mutagenesis as well as its interaction with DNA. The steroid treatment to the bacterial cell/calf thymus DNA/plasmid DNA generates active oxygen radical species. At low dose, it damages DNA, both, in vivo as well as in vitro. In vivo damage leads to the initiation of SOS response and induction of mutagenesis whereas in vitro damage leads to the modification of pyrimidine preferentially in the thymine bases, after or prior to local opening in DNA, with the possible formation of single strand breaks. At higher doses the intensity of DNA damage under in vivo conditions, exceeds the tolerable limit of the cell presumably due also to severe injury to the metabolic machinery which leads to lethality whereas under in vitro conditions the formation of interstrand cross-links as well as the DNA degradation occurs.