There is an interesting evidence that human carcinogenesis is to a large extent the result of exposure to substances present in the environment. These also include life style related factors such as diet, drinking and smoking habits. The methods currently in use are based either on the chemical analysis of human mutagens for the presence of known carcinogens or their metabolites or on the investigation of human material for the presence of genotoxic activity or effects. The rationale for the latter technique is the relationship between genotoxicity and carcinogenicity. During the last decade ample evidence has been obtained that genotoxic events play an important role in the process of carcinogenesis. Therefore, the occurrence of genotoxic effects on DNA of somatic and germ cells of humans and the presence of genotoxic compounds in excreta, secretions and other body fluids have been used as criteria of exposure in
the biological monitoring of carcinogens. Until now, application in practice has been confined mainly to the testing of urine for mutagenicity, carcinogenicity and microscopic examination of lymphocytes for the presence of SCEs and chromosomal aberrations. Besides, other mammalian systems such as micronuclei and Dominant lethal test have provided indirect animal experimental support to the field.

It is known that to unravel the mechanism of induced mutagenicity and carcinogenicity, the role of metabolism and structure of the chemical has got an importance, since the covalent alteration of DNA with the electrophilic metabolites is the common feature in the initiation of these phenomena. By structural alteration or blocking the formation or detoxifying the effective metabolites, the important chemicals can be made risk free. For these reasons it is being stressed to perform studies on the metabolites of the chemicals so as to establish relationship between structure and activity regarding mutagenesis and carcinogenesis.

In the present project, o-toluidine along with 9 of its possible metabolites and 5 metabolites of methyl ester of p-aminobenzoic acid were studied to see their mutagenicity in mice and human lymphocytes. The
parameters used are micronuclei, chromosomal aberrations, sperm head abnormalities, dominant lethality in mice and chromosome aberrations and SCEs in human lymphocytes. The result obtained with these parameter have been summarised.

MICRONUCLEI:
a] o-Toluidine and its metabolites: In the cases of o-toluidine o-nitrotoluene, o-nitrosotoluene, N-acetyl-N-[2 methylphenyl] hydroxylamine, 2,2-dimethyl azoxybenzene, 3,3-dimethyl benzidine and N,N-diacytethyl-3,3-dimethyl benzidine, the values were significant with most of the doses in 6 and 30 hrs treatment. The frequency of micronuclei caused by 2,2-dimethyl azobenzene and N-acetyl [o-toluidine] was significant in the upper one and two doses respectively in 6 hrs treatment.

b] Para-aminobenzoic acid metabolites: Methyl p-nitrobenzoate and methyl p-nitrosobenzoate formed significant micronuclei with all the doses. In the cases of other three metabolites; methyl p-aminobenzoate, 4-carboxy methylphenyl acetanilide and O-acetyl, N-acetyl-4-carboxy methylphenyl hydroxylamine, the frequency of micronuclei was significant only with the upper doses for both the durations.
CHROMOSOME ABERRATIONS IN VIVO

a] o-Toluidine and its metabolites: o-toluidine caused significant aberrations and clastogenicity, [Nos. of break points] with all the doses. In o-nitrotoluene the values were significant for the highest frequency of dose o-nitrosotoluene caused highest chromosome aberration. The frequency of chromosome aberrations was significant in most of the doses with 2,2-dimethyl azoxybenzene and in the highest dose of N-acetyl-2-[2-methylphenyl] hydroxylamine. Other chemicals showed some sort of negative results for this parameter.

b] para-Aminobenzoic acid metabolites: The values were dose response related in the cases of methyl p-nitro and methyl p-nitrosobenzoate. Methyl p-aminobenzate, 4-carboxy methylacetanilide and O-acetyl-N-acetyl methylphenyl hydroxylamine did not cause any significant elevation in the frequency of chromosome aberrations excepting with highest dose in the former two.

SPERM HEAD ABNORMALITIES

a] o-Toluidine and its metabolites: The incidence of sperm head abnormalities in animals treated with o-toluidine and its metabolites along with DMSO [control] was 3.00 ± 0.14 for controls, 3.45 ± 0.24 for o-toluidine, 6.65 ± 0.35 for o-nitrotoluene, 3.85 ± 0.21
for o-nitrosotoluene, 4.25 ± 1.34 for N-acetyl-N-acetoxy-o-toluidine, 3.20 ± 0.28 for 2,2-dimethyl azoxybenzene, 5.10 ± 0.98 for o-toluidine, 6.65 ± 0.07 for N,N-diacyl-3,3-dimethyl benzidine and 6.40 ± 0.56 for N-acetyl-[o-toluidine]. The order of the metabolites in causing the incidence of sperm head abnormalities is 2,2-dimethyl azoxybenzene > o-nitrotoluene > N,N-diacyl-3,3-dimethyl benzidine > N-acetyl [o-toluidine] > o-toluidine > N-acetyl-N-acetoxy o-toluidine > 2,2-dimethyl azobenzene. The t-test shows that these values were insignificant in N-acetyl-N-acetoxy o-toluidine, 2,2-dimethyl azobenzene and in the parent compound o-toluidine; highly significant in o-nitrotoluene, 2,2-dimethyl azoxybenzene and N,N-diacyl-3,3-dimethyl benzidine (p > 0.05) and significant in o-nitrosotoluene, N-acetyl-o-toluidine (p > 0.05) and 3,3-dimethyl benzidine (p > 0.10).

b) para-Aminobenzoic acid metabolites: The incidence of sperm head abnormalities observed in different chemicals was 3.00 ± 0.14 for DMSO [control], 4.75 ± 0.35 for methyl p-nitrobenzoate, 7.20 ± 0.56 for methyl p-nitrosobenzoate, 5.40 ± 0.14 for methyl p-nitrobenzoate, 5.10 ± 0.84 for methyl p-nitrosobenzoate, 5.10 ± 0.84 for 4-carboxy methyl acetanilide and 5.45 ± 0.35 for O-acetyl-N-acetyl-4-carboxy methylphenyl hydroxylamine. The descending
order of sperm head abnormalities caused by these compounds was methyl \( \mu \)-nitrobenzoate > methyl \( \mu \)-nitrosobenzoate > O-acetyl-N-acetyl-4-carboxy methyl-phenyl hydroxylamine > 4-carboxy methyl acetanilide > methyl \( \mu \)-aminobenzoate.

This parameter was analyzed statistically by using t-test between the control and treated groups. The results show that the incidence of abnormal sperm heads increased significantly in the case of all the metabolites \( p > 0.01 \) for all excepting 4-carboxy-methyl acetanilide where it was \( p > 0.05 \).

DOMINANT LETHALS

\( a \) \( o \)-Toluidine and its metabolites: In the experiments it was observed that the lethality varied from 13\% to 100\%. The lethality of the parent compound was 5\%, that was 100 for \( o \)-nitrotoluene, 47 for \( o \)-nitrosotoluene, 38 for N-acetyl-N-acetoxy-\( o \)-toluidine, 60 for 2,2-dimethyl azo benzene, 45 for N-acetyl-N-acetoxy-\( o \)-toluidine, 13 for \( o \)-toluidine, 0 for \( N,N \)-diacetyl, 3,3-dimethyl benzidine and 65 for N-acetyl [\( o \)-toluidine].

The males treated with N-acetyl-N-[2-methylphenyl] hydroxylamine at the same equimolar
concentration of the other metabolites died before the completion of 5 weeks of the treatment. Since it may be inferred that this metabolite was lethargic to the animals even at $10^{-3}$ concentration and caused death to them within 5 weeks of the treatment. The males treated with $N,N$-diacetyl 3,3-dimethyl benzidine failed to impregnate the female mice. The chronic exposure might cause oligospermia or formation of non-mobile sperms in these males.

The gradation of the metabolite with regard to dominant lethality in decreasing order is o-nitrotoluene > $N$-acetyl [o-toluidine] > 2,2-dimethyl azobenzene > o-toluidine > o-nitrosotoluene > 2,2-dimethyl azoxybenzene > $N$-acetyl-N-acetoxy o-toludine > 3,3-dimethylbenzidine.

para-Aminobenzoic acid metabolites: The frequency of lethality (in percent) observed in dams treated with different metabolites of methyl ester of $p$-aminobenzoic acid [PABA] was 7 for methyl $p$-aminobenzoate; 40 for methyl $p$-nitrobenzoate, 57 for 4 carboxy methyl acetanilide and 7 for $O$-acetyl-$N$-acetyl-4-carboxy methylphenyl hydroxylamine. In the case of methyl $p$-nitrosobenzoate, no female became pregnant which were mated with the male treated with this metabolite.
The order of the metabolites to induce the dominant lethality in the increasing rate was:

4-carboxy methyl acetanilide > methyl p-nitrobenzoate > methyl p-aominobenzpate and O-acetyl-N-acetyl-4-carboxy methylphenyl hydroxylamine.

CHROMOSOME ABERRATIONS IN VITRO

a] o-Toluidine and its metabolites: o-Toluidine, o-nitrotoluene, N-acetyl-N-[2 methylphenyl] hydroxylamine, N-acetoxy-N-acetyl o-toluidine 2,2-dimethylazobenzene, 2,2-dimethylazoxybenzene was positive in this test. This test was carried out with only these chemicals.

b] para-Aminobenzoic acid metabolites: Methyl para-aminobenzoate, methyl para-nitrobenzoate, 4-carboxy methyl acetanilide and O-acetyl-N-acetyl-4-carboxy methylphenyl hydroxylamine showed the elevated chromosome aberration over the control in this test. In the latter two, the results were dose-activity related. Methyl p-nitrosobenzoate was not studied in this test.

SISTER CHROMATID EXCHANGES

a] o-Toluidine and its metabolites: The values of SCEs were significant in o-toluidine, N-acetyl-N-[2-methylphenyl] hydroxylamine, 2,2-dimethyl azobenzene, 2,2-dimethyl azoxy benzene, 3,3-dimethylbenzidine. It was dose-response related in o-toluidine, N-acetyl-N-[e-
methylphenyl] hydroxylamine. o-Nitrotoluene and N-acetyl-O-acetyl o-toluidine did not elevate significant SCEs incidence. This test was not carried out with o-nitrosotoluene, N-acetyl [o-toluidine] and N,N-diacyl 3,3-dimethylbenzidine.

b) para-aminobenzoic acid metabolites: The values were significant in the case of methyl 2-aminobenzoate and O-actyl-N-acetyl 4-carboxy methylphenyl hydroxylamine. No experiments were carried out with other 3 chemicals.

On the basis of observation obtained above various schemes have been proposed with regard to the structure and activity of the chemicals.

Like other carcinogenic aromatic amines, o-toluidine in the process of metabolic activation in vivo could also generate proximate or ultimate carcinogenic/mutagenic reactive electrophilic species. A scheme has been proposed for the action of o-toluidine. o-Toluidine in process of N-oxidation could generate -NHOH [hydroxylamine] to its acetyl derivatives -N-OH [hydroxamic acid] and -N-Ac [N-acetyl-N-acetoxy]. The hydroxylamine generated could be further oxidized to o-nitroso or o-nitro-toluene. Aromatic amines when administered are detected in
maximum concentrations as nitrosoarenes in the blood in the first 30 minutes and then the concentration starts falling.

Nitrosoarenes having unsaturated nitroso -N=O function have been considered to be activated electrophilic species and which could react as such with tissue nucleophile or get further biotransformed through enzymatic or non-enzymatic reactions in the presence of various co-enzymes. All the products tested in the present investigation could arise through the primary or secondary metabolic biotransformation of o-toludine [Scheme 1 and 2]. This gets substantiated as o-nitrosotoluene, a metabolic intermediate of o-toluidine on treatment with 1,1-diacetyl, 1,1-4,4- tetrahydro-4-4-bipyridine in a biomimetic model of NAD(P)H and acetyl COA gave all the products enlisted in scheme.

In another scheme it has been proposed that 2,2-dimethyl azoxybenzene, a secondary metabolite which could arise metabolically either from 2,2-dimethyl azobenzene or from condensation of nitroso and hydroxylamine has surprisingly been found to be the most active in sperm head assay at 10^-3 M concentration and in micronucleus test at 10^-2 M, 10^-1 M concentrations after 6 hours treatment and at 10^-1 M concentration with
30 hours treatment. Azoxybenzenes have usually been considered to be the detoxified secondary metabolites arising from the most reactive nitroso and hydroxylamine metabolites. Keeping in view its very high [statistically significant] activity in the present studies, it could be proposed as in reversible pathways in scheme 2, that azoxybenzene via:

[i] azo and hydrazo might rearrange to 3,3-dimethylbenzidine near the target site and which has been reported to be a very potent carcinogen or

[ii] via azobenzene undergoes reductive cleavage to give two moles of well documented carcinogen o-toluidine.

In sperm head assay, it is interesting to observe that both o-toluidine and 3,3-dimethyl benzidine are equipotent. This postulation raises many controversial points as of the many intermediates, 2,2-dimethylazobenzene itself is least toxic in sperm head assay and 3,3-dimethyl benzidine and o-toluidine having equal toxicity are all lesser toxic than 2,2-dimethyl azoxybenzene. Looking into the experimental facts it could only be said that biotransformation of all the compounds from the site of entry to the target site [s] depends upon various factors alongwith
the physical characteristics of the compounds. In the present investigation, it could be concluded that 2,2-dimethylazoxybenzene possibly generates more toxic metabolite(s) at the target site.

o-Nitrotoluene, a starting material for o-toluidine has statistically significant toxicity in sperm head assay and in micronucleus test at certain dose levels in both 6 and 30 hrs. Of treatment. o-Nitrotoluene in vivo appears to be toxic due to possible generation of many reactive metabolites as nitroso, hydroxylamine or esters of hydroxylamine at the target site. o-nitrosotoluene and N-hydroxy-o-toluidine have been reported to be the potent base pair genotoxic compounds in Ames test in presence of S-9 activation system. It has also been found that o-nitrosotoluene and N-acetyl-N-hydroxy-o-toluidine at certain concentrations have significant toxicity in micronucleus test in both 6 and 30 hrs of treatment.

Para-aminobenzoic acid

It is apparent from the data on the possible derivatives of PABA, it is evident that although all the compounds do cause micronuclei, the frequency was not significant with 4-carboxy methyl acetanilide, O-acetyl-4-carboxy [methylphenyl] hydroxylamine and methyl-p-
amino benzoate after 6 hrs. Both methyl-\(p\)-nitrobenzoate and methyl-\(p\)-nitrosobenzoate induced highly significant amounts of micronuclei. In the case of chromosome aberrations, it has been seen that the nitroso compounds yield much more of them than nitro derivatives whereas in the case of micronuclei (both after 6 and 30 hrs.), the values are almost the same. All those three compounds which had produced very low frequency of micronuclei after 6 hours, produced highly significant values after 30 hours. The explanation may be given in the form that the methyl \(\beta\)-aminobenzoate, 4-carboxy methyl acetalilide and O-acetyl-N-acetyl-4-carboxy [methyl \(\beta\)-phenyl] hydroxylamine would have changed into more reactive compounds hitherto unexplained. The values after 6 hrs stood as O-acetyl-N-acetyl-4-carboxy [methyl \(\beta\)-phenyl] hydroxylamine, 4-carboxy-methyl acetalilide at the top and methyl \(\beta\)-aminobenzoate at the bottom.

Methyl \(\beta\)-nitrobenzoate produced highest number of sperms with abnormal heads, the values with methyl \(\beta\)-nitrosobenzoate, 4-carboxy methyl acetalilide, o-acetyl-N-acetalilide, 4-carboxy [methyl \(\beta\)-phenyl] hydroxylamine stood almost equal. The frequency of spermatozoa with abnormal heads produced by methyl \(\beta\)-nitrobenzoate was 7.2 ± 0.56, the value being much higher than that with
other compounds indicating therein that whatever mutations are caused by nitrobenzoate are capable of crossing the barrier of testes and blood much more frequently than the other compounds.

The most striking observations are with regard to the dominant lethals, the data indicate that the aberrations caused by nitrobenzoate were so lethal that they did not allow any impregnation in the females. Although the values with nitrobenzoate were higher still the viability was up to the level of 60%. The lethality of methyl $\rho$-aminobenzoate, O-acetyl-N-acetyl-4-carboxy methyl phenyl hydroxylamine has also been evident. These results signify that although there is quite a bit of variation in the frequency of micronuclei and the spermatozoa with abnormal heads, the lethality of methyl $\rho$-aminobenzoate and O-acetyl-N-acetyl-4-carboxy [methylphenyl] hydroxylamine is almost the same. This may be indicative of the extent of damage caused probably due to the gene manipulations not detected so far.

It is apparent that the net result although $\rho$-nitrosobenzoate produces less amount of micronuclei and less number of spermatozoa with abnormal heads, the ultimate lethality towards embryos is maximum indicating
therein the more genotoxicity of this compound over nitro proving therein the contention about the high chromosome aberrations producing capacity of this compound over nitro-nitrogroup. It is now generally believed that nitro compounds on metabolic activation change into nitroso i.e. \( \text{NO}^2 = \text{NO} \) which in turn change into \( \text{NHOH} \). There is no doubt that the pathway is from nitroso and then to hydroxylamine, but more reactions probably are leading towards detoxification when it reaches to amino derivatives. It is quite evident from the current observations that dominant lethality with nitro is 40%, whereas with nitroso, it is 100% and as soon as it reaches to hydroxyl and amino it comes to 70% and that is probably the reason that PABA as such has been found to be anti-mutagenic in many of the test systems worked out by various authors. In case the cells are attacked by nitroso or hydroxyl compounds, they get transformed but if there is any machinery which can accelerate these reactions, probably loss of mutagenicity can lead to loss of carcinogenicity and it is quite possible that it is on this principle that the anticarcinogenicity of PABA is based i.e. when it comes in contact with another carcinogen, its detoxifying reactions are accelerated beside detoxifying the carcinogenicity. This hypothesis needs further
verification and observations are on in this direction.

Methyl ester of \( \mu \)-aminobenzoic acid and its primary metabolites have been used to increase the lipophilic character of these compounds. The methyl esters \emph{in vivo} can be easily converted to the corresponding acids by the ubiquitous presence of enzymes esterase. Methyl \( \mu \)-nitrobenzoate a starting material for methyl \( \mu \)-aminobenzoate has statistically significant toxicity in sperm head assay, micronuclei test at almost all dose levels with 6 and 30 hrs treatment. In dominant lethal test also, it inhibited 40% implants with respect to control.

Chemically the aromatic nitro compounds as such are unreactive towards nucleic acids and proteins and thus the toxicity of methyl \( \mu \)-nitrobenzoate could not be considered due to the compound itself, but possibly due to other highly reactive electrophilic metabolite[s]. As seen in scheme 4, the carcinogenic aromatic amines via N-oxidation and aromatic nitro compounds via nitro reduction could generate reactive nitroso, hydroxylamino or esters of hydroxylamines as proximate/penultimate or ultimate electrophilic carcinogenic species. The methyl \( \mu \)-nitrobenzoate which itself is chemically unreactive seems to generate chemically
activated species near the target site and, thus, shows the maximum toxicity among the series of the compounds tested.

Aromatic nitroso compounds when generated in situ unlike the nitro derivatives react directly with the tissue nucleophiles and have been reported to be more toxic. But in the present investigations on the contrary, methyl α-nitrosobenzoate has been found to have lower toxicity in sperm head assay and in micronucleus test.

The toxicity of a reactive metabolite at any tissue site would be expected to be dependent upon its concentration and the site of action. Nitrosoarenes being reactive as such on administration are expected to get sequestered in part and then lost before reaching to the action site i.e. the sperm or the bone marrow and thus may be available in lower concentration than the dose at the active site administered. However, nitroso derivatives in the dominant lethal test, were found to be the most toxic as no implant was observed, N-acetyl-N-acetoxy derivatives of many carcinogenic aromatic amines have been considered to be ultimate carcinogenic species [Miller and Miller, 1969]. But the N-acetyl-N-acetoxy derivative of methyl
$\alpha$-aminobenzoate in the present investigations has been found to have lower toxicity than the corresponding nitro and nitroso derivatives. In dominant lethal test, its toxicity is even much lower than N-acetyl derivative of methyl $\alpha$-aminobenzoate. Being highly reactive, N-acetyl-N-acetoxy derivatives of carcinogenic aromatic amines are normally more toxic at the site of administration than at the distant organs and this could also explain the lower toxicity of this derivative in sperm head assay and micronuclei test systems. The toxicity of N-acetyl derivative of methyl $\alpha$-amino benzoate and possibly be considered via N-hydroxylation and their further conversion to reactive esters as seen in scheme 3.