Chemical mutagenesis: From plants to human

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Following the discovery that chemicals, like ionizing radiation, can induce mutations in *Drosophila* in the 1940s, work on chemical mutagenesis was started in many parts of the world. At the Botany Division of Indian Agricultural Research Institute, chemical mutagenesis studies in crop plants were initiated by M. S. Swaminathan in the 1950s. This brief review deals with progress of chemical mutagenesis studies, which started with plants and has been extended to rodents and humans over the years, in which I have had the privilege to participate.

Keywords: Chemical mutagenesis, *Drosophila*, mutations, biomonitoring.

FOLLOWING the pioneering radiation studies of Muller in *Drosophila*[1] and Stadler in maize[2], the discovery that chemicals can induce mutations in *Drosophila* and plants was made in the USSR and United Kingdom during 1940s and came to light after the second world war[3,4]. Similar to ionizing radiation, using chemicals to induce mutations in plants for breeding purposes became popular, especially in Sweden. In the 1950s, in addition to radiation mutagenesis, chemical mutagenesis in plants was initiated by M. S. Swaminathan at the Botany Division of the Indian Agricultural Research Institute. Besides producing mutations in crop plants of agronomic value, basic studies on the mechanisms of induction of mutations and chromosome aberrations were also carried out. In this review, an attempt is made to summarize the results of earlier studies in plants started in India, which lead to further research in *Drosophila* and mammals including human.

Vegetable oils – Novel chemical mutagens

After the nuclear bombing with devastating effects on the populations of Hiroshima and Nagasaki, great efforts were made to use atomic energy for peaceful purposes, especially by the Western nations led by the United States. One of such efforts was to use ionizing radiation as a tool to induce useful mutations in crop plants. Under the Second Five-year Plan of the Government of India, a project to use radioactive isotopes in agriculture research was granted and I had the privilege to be appointed as a research assistant in that project. Many of the crop plants, such as wheat, rice, maize, barley, rape, etc. were treated with either radioactive isotopes (^{32}P, ^{35}S), X-rays or fast neutrons to induce mutations. One of the earliest observations made under that project was that among the different crop plant species studied, there was big variation in their sensitivity to radiation, oil seed plants being more resistant in comparison to cereals. It was thought that the oil present in the seeds bestowed a protection against the lethal effects of radiation. Therefore, we soaked seeds of different cereals in various vegetable oils, such as, mustard oil, groundnut oil, hydrogenated groundnut oil (vanaspathi), castor oil, linseed oil, coconut oil, gingelly oil and ghee, prior to X-irradiation. In these experiments, controls were treated with the oils alone. To our great surprise, some of the vegetable oils were found to be extremely potent in inducing chromosome aberrations and the results were published in *Current Science*[5]. Groundnut and mustard oils were the most potent ones, whereas gingelly oil and ghee were least potent in inducing chromosome aberrations. In addition, some oils, such as castor oil was able to disrupt the spindle, thus making chromosome spreading easy[6]. These studies were extended to check whether the vegetable oils could induce mutations in crop plants such as diploid, tetraploid and hexaploid wheats. While chromosome aberrations were observed in the root meristems and pollen mother cells of all the three species of wheat, mutations were observed only in hexaploid wheat[7]. These results indicated that vegetable oils induce only chromosomal mutations, namely deletions and duplications, which are tolerated in an hexaploid species and not in diploid and tetraploid species. Vegetable oils act through an early intermediate, perhaps a free radical formed in the autooxidation process[8]. We pointed out the possible implications of our observations on the mutagenic properties of vegetable oil towards human health, especially diet-related cancer. Though the importance of our work was not recognized at that time, a survey of the current literature shows that the importance of dietary intake of fat and its relation to colorectal cancer, prostate and breast cancer is being recognized to a great extent[9]. It has recently been shown that some vegetable oils, such as flax oil are genotoxic as evaluated in *Drosophila* wing spot test (indicator of somatic recombination), whereas some oils like virgin olive oil is nongenotoxic[10].

Alkylating agents

Studies with higher plants

Alkylating agents (AA) are potent mutagens and can be classified broadly into monofunctional and bi- or poly-

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functional ones, depending upon the number of alkyl groups present in the compound. The first chemical tested at the Indian Agricultural Research Institute was nitrogen mustard, a bi-functional alkylating agent[11]. However, systematic studies on different crop plants using AAs were initiated by Swaminathan and his students in the late fifties[12].

From the pioneering studies of Ehrenberg and coworkers in Sweden[13,14] it was clear that AAs are particularly suited for mutagenicity studies in plants. Thus, in-depth studies employing different AAs were started by me and my students to correlate various biological effects, such as killing, induction of chromosomal aberrations and mutations with their chemical reaction patterns[15–17]. The reactivity of AAs towards nucleophiles can be defined in terms of reaction mechanisms and the dependence of reaction rates on the nucleophilic strength of the receptor atoms[18,19]. An useful expression of the reactivity of AAs is Swain-Scott substrate constant *s*, which is the measure of sensitivity of AA to the strength *n* of the nucleophile with which it reacts[18]. Two principal types of nucleophilic substitution reactions have been invoked. The reaction types are generally referred to as unimolecular (*SN1*) and bimolecular (*SN2*) (for a detailed discussion, see ref. 20). The ability of various alkylalkanesulfonates (such as, methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), isopropyl methanesulfonate (IPMS)) to alkylate various sites in DNA was found to vary in accordance with the expectations based on *s* values[18]. The most common adducts in DNA alkylated in neutral solution was 7-alkylguanine[21]. However, the proportional extent of reaction at the N-7 position varied according to the *s* values, the high *s* value of the AA was correlated with high N-7 alkylation. Conversely, the alkylation of O-6 alkyl guanine was higher for AAs with low *s* values. The biological effects of different AAs were found to be correlated with the *s* values of the AA employed. For example, in barley, AA with high *s* value (MMS) was found to be more cytotoxic and less mutagenic in comparison to an AA with low *s* value (propyl methanesulfonate, PMS)[19]. Natarajan and coworkers[15–17] studied extensively the frequencies of chromosome aberrations induced by different alkylalkanesulfonates in both mitotic and meiotic cells of barley. They found that AAs with low *s* values (EMS, butyl methanesulfonate – BMS and PMS) were poor inducers of chromosomal aberrations in comparison to those with high *s* values (MMS and methyl ethanesulfonate (MES))[15]. Higher chromosome breaking ability of MMS in comparison to EMS was also found in studies employing root tip cells of *Vicia faba*[22]. In a comparative study of different alkylalkane sulfonates, Rao and Natarajan[15] concluded that the efficiency of induction of mutations (chlorophyll and viable) was of the following order (decreasing sequence): EMS, EES (ethyl ethanesulfonate), BMS, PMS, MES and MMS. The results from studies using other plant systems such as *Arabidopsis thaliana* confirmed the above conclusions[23].

Studies with Drosophila

One of the difficulties in making comparisons between different alkylating agents is that the exposure concentration is not an adequate parameter to compare the biological effects because of large variations in the rates of absorption, distribution in cells and tissues and kinetics of inactivation. Accurate dose measurements at the genetically significant targets are possible only when labelled mutagens can be used. Such studies using labelled EMS were initiated by Ehrenberg and myself as early as 1960, but continued in Leiden later in seventies[24,25]. Except for EMS, such information is not available, hence a detailed study was undertaken by Vogel and Natarajan[26,27] in *Drosophila*, using the percentage of recessive lethal mutations as the biological dosimeter of the extent of interaction of AAs with target DNA in germ line. With this procedure, we could compare (a) the proportion of chromosome aberrations (translocations between chromosomes 2 and 3) to the recessive lethals (T/M ratio) at different mutation frequencies, (b) the lowest concentration, expressed as frequency of recessive lethals at which chromosome aberrations are induced and (c) production of recessive lethals relative to cytotoxicity. In these studies[26,27] eleven monofunctional AAs, with *s* values ranging from 0.26 (ethylnitrosourea – ENU) to 0.86 (MMS, dimethylsulfate – DMS) were compared. Some general conclusions could be drawn from the studies in *Drosophila*, namely, (a) two parameters, i.e. dose (intensity of alkylation) and reaction pattern (distribution of alkylation) are important in determining the quality and the frequency of genetic damage, (b) a direct relationship between chromosome breaking efficiency and *s* value and a general inverse relationship between this parameter and the ability of AAs to induce point mutations and (c) the tendency of AAs with low *s* values to preferably induce point mutations is paralleled by low cytotoxicity. These general conclusions confirm the earlier ones derived from studies on higher plants.

Studies with mammals

In vitro studies. In cultured mammalian cells, several biological end points can be studied, which include, cell killing, structural chromosome aberrations, micronuclei (derived from a lagging chromosome break or a whole chromosome during anaphase), sister chromatid exchanges (exchanges between the sister chromatids occurring during the DNA synthetic phase, possibly representing a recombination event) and mutations (derived from single base change or deletions). Following the studies with higher plants and *Drosophila*, experiments were carried out with Chinese hamster cells in culture, using several monofunctional AAs (namely, ENU, methyl nitosourea (MNU), EMS, MMS and DMS) with different *s* values ranging from 0.26

(ENU) to 0.86 (DMS, MMS)[28]. In this study, molecular dosimetry using tritium labelled ENU and EMS was also carried out, which showed that induction of point mutations in the *HPRT* (hypoxanthine-guanine phosphoribosyl transferase) locus was directly correlated with the induction of O6-ethylguanine. The induction of point mutations was not correlated with the induction of cell killing, chromosomal aberrations and sister chromatid exchanges. The general pattern of response was similar to that obtained with plants and *Drosophila*, namely that AAs with high *s* values are more efficient in induction of cell killing and chromosomal aberrations whereas the AAs with low *s* are potent inducers of point mutations.

In vivo studies. Several AAs have been studied in mammals, especially in mice, using different end points, such as micronuclei in polychromatic erythrocytes in bone marrow or erythrocytes in blood cells, chromosomal aberrations in bone marow cells, mutations in splenocytes, heritable translocations, multivalent formation in spermatocytes, dominant lethals, sperm abnormalities, specific locus mutations for coat colour, etc. However, it is difficult to make valid comparisons in view of the inherent differences in the metabolism and distribution of mutagens *in vivo*. For evaluation of chemicals of importance with regard to human exposure, such as ethylene oxide, 1-3-butadiene, methods to estimate mutant frequencies in lymphocytes or splenocytes of rodents exposed *in vivo*, a clonal assay was standardized in our laboratory[29,30]. In this method, frequencies of mutations in the *HPRT* locus are determined by the number of 6-thioguanine-resistant clones against the controls. The advantage of this approach is that it allows parallel determination of DNA adducts, hemoglobin adducts in the erythrocytes as well as other parameters such as chromosomal aberrations, SCEs and micronuclei. Such data are useful for estimation of genetic or carcinogenic risk due to exposure to specific chemicals in human (see below).

Metabolic activation of chemical mutagens

Many chemical mutagens and carcinogens do not act directly by inducing lesions in DNA, but have to be metabolically activated by the cellular enzymes. This category of chemicals includes AAs like dimethyl nitrosamine (DMN), diethyl nitrosamine (DEN), and polycyclic aramotic hydrocarbons (PAH) such as benzo(a)pyrene, 1,1 dimethyl-1,2-benzanthracene (DMBA). Ames and coworkers[31] developed an *in vitro* mutagenicity detection system using the bacteria *Salmonella* and rat liver microsomes (S9 fraction), popularly known as Ames's test. This methodology was adapted for detecting mutagenic effects of indirectly acting AAs in cultured mammalian cells and standardized by Natarajan and coworkers[32]. Ideally, it would be very practical in *in vitro* studies, if one can use the same cells for activitating as well as target cells for detecting biological effects. This was achieved by using human hepatoma cells (Hep G2) which have the capacity to activate most of the indirectly acting mutagens and several biological end points can be studied in the same cells[33,34] without employing any external metabolic activation system.

Biomonitoring of human population exposed to chemical mutagens/carcinogens

Human populations are continuously exposed to mutagenic/carcinogenic chemicals, either environmentally or occupationally. The monitoring of genotoxic effects of carcinogens in humans is increasingly applied for hazard identification and risk assessment purposes, which requires a multi-disciplinary approach[35]. International organizations, such as International Commission for Protection against Environmental Mutagens and Carcinogens (ICPEMC)[36], and International Programme on Chemical Safety (IPCS)[37] have developed standardized protocols for such studies. Occupational exposure to carcinogens, such as, vinyl chloride, ethylene oxide, 1,3-butadiane, chemothereupetic agents leading to detectable biological effects is well documented. Massive human exposure to arsenic, a known human carcinogen, in the form of drinking water in West Bengal, Bangladesh, Taiwan, Thailand and Latin America is recognized. Accidental exposure of a large population to chemicals, such as methyl isocyanide (as it happened in Bhopal, the biological effects on exposed individuals were not, unfortunately, adequately investigated – a missed opportunity) do occur in various parts of the world, but the impact in exposed populations is not properly evaluated. I have been involved in monitoring human populations exposed to chemical carcinogens and ionizing radiation for a long time, where my basic cytogenetic skills gained in India became useful.

One of the first reports on the occurrence of chromosomal aberrations in workers occupationally exposed to a chemical carcinogen was about a cohort in Norway exposed to vinyl chloride[38]. Now, it is recognized that there are many biomarkers, such as DNA adducts, hemoglobin adducts, chromosomal aberrations, micronuclei, SCEs and mutations in lymphocytes, which can be used to assess the extent of exposure to a genotoxic agent. Among these, the only biomarker which has been shown to be correlated with future outcome of cancer in human population has been the chromosomal aberration[39]. A comprehensive biomonitoring study of workers occupationally exposed to ethylene oxide, a chemical which has been classified as a human carcinogen (category 1) by International Agency for Research on Cancer (IARC), used as intermediate in the production of ethylene glycol, non-ionic surfactants and other derivatives as well as for sterilization of medical devices, was carried out by us[40]. In this study, the frequencies of hemoglobin adduct –N(2-hydroxyethyl valine (HOEtVal) in the erythrocytes, chromosomal aberrations,

micronuclei, SCEs and *HPRT* mutations in the peripheral blood lymphocytes from a group of workers exposed to high and low levels of ethylene oxide and matched controls were determined. Among the biomarkers studied, hemoglobin adduct was the most sensitive indicator of exposure, as this gives the cumulative value for the last four months of exposure, which is the average life time of erythrocytes in human. These adducts, unlike DNA adducts, are not subjected to repair. Among the biological end points, SCEs and chromosome aberrations came out as sensitive indicators of exposure[40]. The importance of these studies as well as those done in rodents is the possibility to use these data to make a risk estimate for heritable effects. There are two methods proposed for such a risk estimation, namely, the parallelogram approach suggested by Frits Sobels and rad-equivalent approach proposed by Lars Ehrenberg[41].

With the parallelogram approach, it could be concluded for ethylene oxide, exposure for one working year (1800 h) to 1 ppm, would lead to an incremental risk of 4×10^{-4} *above the background* that a disease with dominant inheritance would be transferred to the offspring[41]. With rad-equivalent approach, 1 ppm of ethylene oxide gives rise to a gonad dose[42] of 0.15×10^{-3} mMh corresponding to $6(3–12) \times 10^{-3}$ rad equivalents. These approaches give only an approximation of the risk associated with the exposure to a known mutagen/carcinogen. However, there are indications that genetic polymorphisms, for enzymes involved in metabolism, and DNA repair, may have an influence on individual susceptibility[43].

Studies with arsenic

Arsenic (As) is classified as a human carcinogen. At present, millions of humans all over the world are chronically exposed to As at sufficiently high levels to cause severe toxic effects, including cancer. Occupational exposure to As is associated with smelting of copper sulphide ores and in mining and production of gold, tin, tungsten, lead and zinc. However, the most extensive exposure to As occurs in developing countries, like, Argentina, Chile, India and Bangladesh, where drinking water from wells is contaminated with high levels of As. Inorganic As does not directly interact with DNA but interferes in DNA repair and replication under *in vitro* conditions[44]. Our initial biomonitoring studies were conducted in Bulgaria in a small population living in the close vicinity to the copper smelter at Srednogorie, where an increase in the frequencies of micronuclei in the lymphocytes was observed. However, effect of exposure to other heavy metals, especially selenium, could be a confounding factor[45]. In order to avoid mixed exposures, subsequent efforts were directed towards monitoring populations in an area that is devoid of industrial pollution where As intake occurred solely by drinking water. These studies were mainly conducted at four sites in Salta Province of Northwestern Argentina, including San Antonio de la Cobres and Salta Forestal in the Western part of Salta Province as well as Taco Pozo in the province of Chaco, with Rosario de Lerma situated close to Salta city as control area[46]. The average As levels in drinking water ranged from 0.7 μg/l (control area) to 205 μg/l. The As contents in urine, blood as well as the frequencies of micronuclei (MN) using (cytokinesis block technique) and chromosomal translocations in the lymphocytes, were determined. The highly exposed populations had higher frequencies of MN, five to six fold increase, over the controls. In spite of very high concentration of As in San Antonio de la Cobres, the exposed population which was exclusively of native Indian origin had no indication of ill health or skin lesions, whereas those from other areas, such as Chaco region, who were mainly mestizos, had typical symptoms of As toxicity. The difference in response to As between different ethinic groups appears to be due to the pattern of metabolism of As. The native Indians were found to exhibit different metabolic dispositions than those found for populations in Europe, US and Japan. These Indians excrete As DMA (dimethylarsinic acid) and inorganic arsenic with very little monomethylarsonic acid (MMA), indicating that they detoxify As very efficiently[47].

In West Bengal and Bangladesh it has been estimated that millions of humans are exposed to high levels of As in drinking water. We have been collaborating with Giri at the Indian Institute of Chemical Biology, Kolkata, whose group has carried out extensive biomonitoring studies in this population in West Bengal[48–51]. These studies included, determination of As content in finger nails, hair follicles, blood, urine, As metabolites in urine, micronuclei frequencies in three types of cells, namely, lymphocytes, uroepithelial cells, buccal mucosa cells, sister chromatid exchanges and chromosomal aberrations. Several interesting and important observations came out of these studies, which include (a) not all individuals exposed to As exhibit toxicity symptoms, namely several types of skin lesions (hyperkeratosis, hypokeratosis, skin cancer, etc.), indicating a genetic variability among this population which warrants an in-depth study on the existence of genetic polymorphism for enzymes involved in metabolism and DNA repair, (b) both symptomatic and non-symptomatic individuals show high frequencies of MN in all three types of target cells and (c) lymphocytes from As exposed individuals are more resistant to *in vitro* treatment with As (adaptation) in comparison to the lymphocytes from unexposed individuals. This unique population offers immense possibility for basic and applied research, which should be exploited, as the results obtained will be beneficial in the future health care of the exposed As populations and their progenies.

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