# PART 3

# BIOLOGICAL SCIENCES


Simple double-strand break
Complex lesion
Photon
OH
H
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Chappad
Kerikkad
Kayamkulam
Anattupuzha
Alappad
Panmana
Chavara
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# A Historical Perspective of Radiation and Life Sciences

The discovery of X-rays by W. K. Roentgen in 1895 marked the beginning of modern era in physics. The discovery of natural radioactivity in uranium and radium soon thereafter led to almost explosive activity in the field culminating in the development of both nuclear weapons and nuclear power. X-rays found immediate application in clinical medicine for diagnostic purposes. However, when scientists and workers associated with research on radioactivity, including the famous Antoine-Henri Becquerel, developed radiation related problems like skin lesions from radium carried on their person, the possibility of deleterious effects of radiations started manifesting. It is this dichotomous nature of radiations which made them an interesting topic of study. Interaction of radiation with matter, inanimate and animate alike, occupied centre stage of research all over the world. As early as 1927, Prof. H. J. Muller showed that X-rays could cause mutations, i.e. changes in heritable characteristics, in the fruit fly. These effects of radiations, confined to the laboratory till then, were revealed to the world at large most dramatically with the dropping of Fat boy over Hiroshima in August 1945. The survivors continued to provide important data on the nature and extent of radiation induced damage. Following the famous “megamouse” experiment in which thousands of mice were exposed to radiation to study induction of mutations, investigations on the impact on life span of animals due to external exposure as well as ingestion of radioactive material were undertaken. Similar studies were also initiated on other living organisms like plants and microbes.

In 1955 the United Nations Scientific Committee on Effects of Atomic Radiation (UNSCEAR) was constituted to estimate the exposure of human population from various sources of ionizing radiation, both natural and man-made, e.g. radioactive fall-out from nuclear weapons tests, and the ensuing potential health risks like cancer and hereditary effects. In addition, availability of radiolabeled biomolecules provided the much needed sensitive tracers to delineate pathways of a myriad biochemical reactions underlying physiological processes.

These studies brought about a paradigm shift in the conduct of research in life sciences as well. From being what was essentially an observational science, biology soon graduated to become an analytical science much as its physical sciences siblings. It also dawned on everyone that our understanding of the effects of radiation would not get honed unless there was better understanding of the native unexposed, and hence undamaged, living system. Research in basic biology progressed gradually from the study of the external features of the whole organism (morphology) to inner working of its constituent components (physiology) to the study of single units like cells (cell biology) to ultimately the structure and function of constituent molecules (molecular and structural biology). Investigations on regulation of gene expression received an impetus with the introduction of cell lines in animal research that significantly facilitated understanding of the cellular defects leading to development of cancer and the contribution of radiation to this process. The basic science research programmes all over the world received funding from the Department of Energy of the US; notable among them being the Human Genome Project, basically aimed at understanding the nature of genes and genetic basis of disease in man.

Interest in biological research is on the rise today, especially due to introduction of new tools of genomics and proteomics. The effects of acute and chronic radiation exposure of different living systems is of even greater interest, especially now, since the world may have to depend on nuclear power because of dwindling natural non-renewable resources.

**Research in Basic Biology in DAE**

From the time of its inception, the DAE had kept itself abreast of developments in the world at large and undertaken various research programmes in biology. Though the initial interest was confined to research on interaction of radiations with biological matter (Radiobiology) it was recognized very early that this would not become meaningful in the absence of a broad-based, bio-medical research activity of its own. However, the real beginning of basic research in biology at the cellular level was made in the mid-fifties under the guidance of late Dr. A.R. Gopal-Ayengar. The laboratories were housed at the then Indian Cancer Research Centre attached to the Tata Memorial Hospital. It gathered momentum with cytologists such as Dr. K.C. Bora, some biochemists such as Dr. M. B. Sahasrbuddhe, Dr. A. Sreenivasan and food

technologists such as Dr. P. B. Mathur joining the team. The laboratories moved to the Richardson & Cruddas building in south Mumbai blossoming into the Bio-Medical Group of Atomic Energy Establishment, Trombay with Dr. Gopal-Ayengar assuming charge as its first Director. In the early years several interesting research papers were published in journals that would be the envy of the best of the biologists today.

Programmes involving use of radiation for developing newer crop varieties, enhancing the shelf life of food materials, sterilization of medical products and disease diagnosis and therapy have been actively pursued in DAE along with basic research programmes. The success of biological research programme of DAE lies in strong basic groups undertaking research in genetics, molecular biology, biochemistry and radiobiology. These research programmes have paid rich dividends in terms of contributions to national economy as well as in keeping pace with modern developments such as genomics and proteomics. DAE-aided institutions such as Tata Institute of Fundamental Research (TIFR), Cancer Research Institute (CRI), Saha Institute of Nuclear Physics (SINP) and recently established National Centre for Biological Sciences (NCBS) have also made their mark through important scientific contributions in molecular and cell biology, structural biology and cancer research.

# Mutation Breeding

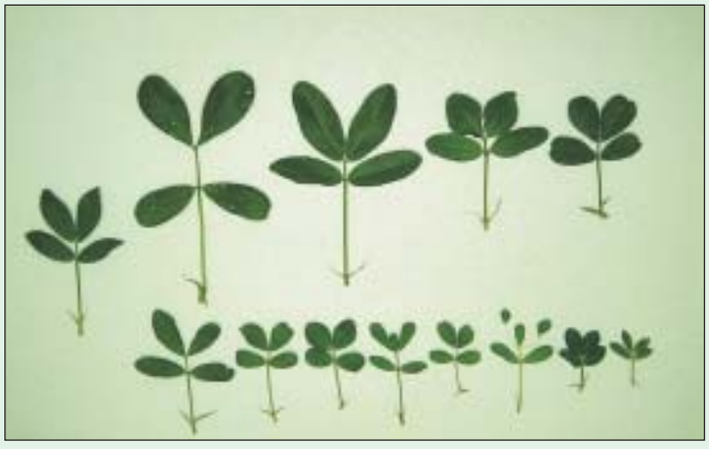
## Link B1

*The global biodiversity around us is the result of accumulated spontaneous mutations in all living organisms. Mutations are the driving force of evolution. They arise either due to damage to DNA bases, which is not corrected properly by the cellular DNA repair machinery or due to mistakes in DNA replication, which escape proof reading or both. On an average, the spontaneous mutation rate is one per locus in a million cells per generation. H. J. Muller showed that exposing fruit flies to X-rays could enhance mutation rates in them. Soon after, similar observations were made by L. J. Stadler who demonstrated that exposing seeds to X-rays enhanced mutation rates in maize. Following these discoveries Swedish and German scientists carried out extensive experiments to establish that radiation-induced mutations can be used for developing improved cultivars of crop plants. Though the process of induction of mutation was a random one and could affect one or more traits, mutants with desired characters could be selected and the specific character could be transferred to the crop variety under cultivation using conventional breeding. Thus a new branch of science called Mutation Breeding slowly evolved that yielded crop plants with several novel and desirable qualitative as well as quantitative traits. These included higher yield due to increased number of pods per plant or number of seeds per plant, disease resistance, oil content, insect resistance, protein quality, maturity period, ability to grow under different soil conditions etc. These mutants could then be used for genetic studies as well as for developing improved cultivars of crop plants. Introduction of such genetically improved varieties globally helped meet the rising demand for food in the face of the ever increasing population and decreasing cultivable land and water resources. It has thus helped many nations override dire Malthusian predictions.*

# Radiation induced variability in morphological characters of crop plants

*Variation in height of groundnut plants*

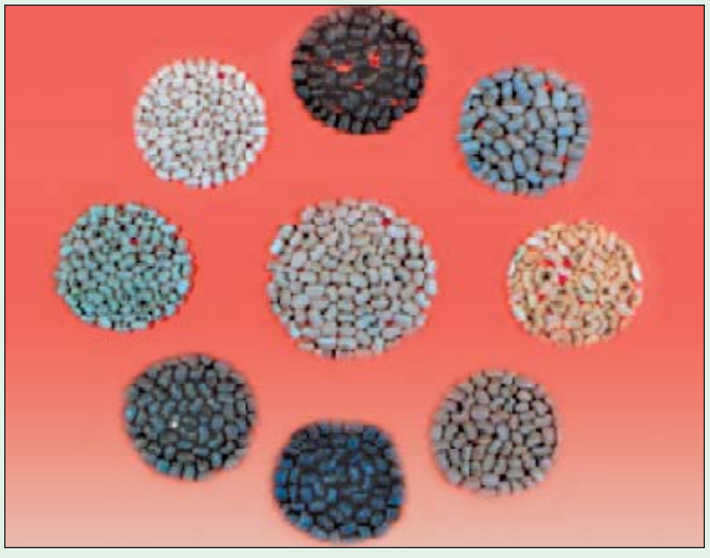
*Dwarfism in sunflower plants*



*Variation in leaflets of groundnut*

*Variation in leaflets of pigeonpea*

*Seed size variation in groundnut*



*Seed colour variation in cowpea*

# Mutation Breeding

## Success story of plant breeding

After the end of the Second World War, study of effects of ionizing radiations on living systems was an area of global interest. Animal cytology was not well developed at that point of time. *Vicia faba*, a leguminous plant with large chromosomes, thus became the obvious material of choice for investigating radiation effects on chromosomes. Seed radiation biology was of considerable interest as seeds were convenient to use as model systems for investigating effects of oxygen, fractionation of doses, radiation sensitizers and protectors. At the Atomic Energy Establishment, now known as the Bhabha Atomic Research Centre, Trombay, Dr. K. C. Bora and N. S. Rao, both cytogeneticists, initiated research on rice and jute respectively. Soon an experimental field station was established to grow plants to maturity from radiation-exposed seeds and to check the progeny for visible mutants. Dr. N. K. Notani initiated experiments for evaluating radiation effects on transposable elements in maize. Radiations were used to create variability in different crop plants, especially in those where natural variability did not exist. This variability in genetic characters yielded mutants, which were used in breeding programmes to develop high yielding varieties of oil-seeds and pulses. Several varieties of groundnut, mungbean blackgram, pigeonpea and soybean have been released by national and state agencies. In this effort some State Agriculture Universities (SAUs) also actively participated. Recently, new technologies such as molecular markers and computer vision are being developed to assist efforts in plant breeding.

Mutations in the second generation after exposure to radiation were identified.The desired ones were selected, tested and developed as varieties that could be handed over to farmers for cultivation. Thus mutant cultivar TR-1 of rice was selected and subsequently released in Kerala under the name Vellayani. Research efforts in groundnut yielded a large kernel mutant cultivar Trombay Groundnut - 1 (TG-1), and a high yielding cultivar TG-3.

A Gamma Garden was established at Trombay for exposing growing plants to chronic gamma radiation. Different tropical fruit trees and shrubs were planted in a large sector of the Gamma Garden with the aim of isolating desired mutants in the perennial species.

### Where is the mutation breeding technique relevant?

- Natural genetic variability is low
- Removal of undesirable trait from elite variety
- Shortening of duration for fixing the trait
- Breaking the linkage between tightly linked genes
- Specific intervention in known biochemical pathways
- Novelty value of a phenotypic change
- Generating variability through recombination is not possible e.g. vegetatively propagated crops

### Development of mutant cultivars

- Identification and stabilization of a desired mutant from the progeny of radiation exposed seeds or other plant parts is the first step.
- After initial evaluation for yield at BARC, these mutants are further evaluated at other locations in a series of field experiments under the Coordinated Research Trials of the ICAR or in the trials conducted by the SAUs.
- If the new selections perform better than the best available cultivar (Check variety), it is evaluated in farmers' fields along with the check variety.
- The Central Varietal Release and Notification Committee of the Ministry of Agriculture, which releases and notifies the new variety, considers the compiled data.
- A similar procedure with some minor variation is followed in the states for varietal release. This is followed by three stages of seed production – breeder, foundation, and certified or truthfully labeled seed, which is finally sold to the farmers.
- The overall procedure takes anywhere between 10-12 generations before the new selection reaches the farmers.
- More often, the mutant is advantageous only in one or two traits such as early maturity, larger seed size or higher oil content but its overall yield turns out to be lower than the parent cultivar or the best check. The mutant is then hybridized to the parent or other cultivar and the desired segregants are selected.

**A new breeding cycle starts**

In the late sixties, fertilizer responsive cultivars of rice and wheat, developed using semi-dwarf plant type mutations, had established their high yield potential. They were gaining popularity, thanks to support of vast resources and research inputs from International Institutes, besides the large network of Indian Council of Agricultural Research (ICAR) and State Agricultural Universities.

Though it is easy to induce mutations by exposure to radiation, the overall procedure of developing a reliable cultivar with desired mutation is a long drawn process. The challenge, therefore, for a small group of less than one dozen scientists at BARC, with limited experimental field area, was to make a lasting impact on the national agricultural scene, and take benefits of nuclear applications to farmers. This dictated that the programme had to be focussed on a relatively small but nationally relevant area. The decision was, therefore, taken to intensify research efforts on oilseeds and pulses. A programme for chromosome engineering (inducing translocations) in wheat was also initiated. Different attributes were selected in different crop plants. These included yield, resistance to biotic and abiotic stresses, qualities like seed size, color etc. This effort has resulted in the release of 25 different varieties of groundnut, pulses, oils seeds, rice and jute.

### Spread of Trombay groundnut varieties in India

Among the released TG varieties, TAG-24 and TG-26 in normal seed size class and TKG-19A in large seed size class are very popular. The share of TG varieties in annual national seed indent is around 25%. Currently, TAG-24 is used as a national check for *rabi*/summer trials while TKG-19A, TG-26 and Somnath are used as check varieties in the respective national and state varietal trials. The high yielding ability of TAG-24 and TG-26 is exploited by farmers by harvesting up to 5,000 kg/ha with TAG-24 during *kharif* season and up to 9,000 kg/ha in 115 days under irrigated *rabi*/summer situation using TG-26, compared to the national average of about 1,000 and 1,500 kg/ha in the respective seasons. In the course of time, several farmers have become spokespersons and seed suppliers of TG varieties in Maharashtra, Karnataka, Andhra Pradesh and Gujarat, leading to vertical and horizontal spread of TG varieties across the country.

## Oilseeds

### Trombay groundnut

In general, genetic variability in groundnut is meagre. Among indued mutants there were two high yielding Trombay groundnut varieties, TG-1 and TG-3. TG-1 is large seeded and TG-3 has more number of branches. Isolation of these agronomically superior mutants gave a new direction to research. Since then, radiation induced mutants are utilzed as one of the parents in hybridization to develop newer TG cultivars. This enables combining superior agronomic traits such as early maturity, larger seed size, high oil content, high shelling percentage, increased harvest index, moderate seed dormancy and tolerance to pests and diseases with higher yield. New mutants or mutant derivatives thus developed were further evaluated in national trials. The first TG mutant variety and the first TG mutant derivative commercially released were TG-1 under the name 'Vikram' and TG-17, respectively. As of today, 10 TG varieties have been released for commercial cultivation.

### Trombay mustard

Rapeseed-mustard is the second most important oilseed crop in India, after groundnut. It accounts for 25% of the area under cultivation as well as total production. Its genetic improvement programme was initiated in the late 1960s. First Indian yellow seed coat mutant was isolated and used extensively in a cross breeding programme. A large number of high yielding yellow seed coat lines were developed.

These efforts have resulted in the development of two high yielding varieties, namely TM-2 and TM-4. Both varieties were released for North Eastern Hilly (NEH) Region of Assam in 1987. TM-2 is a radiation induced direct mutant isolated from parent RL-9 whereas TM-4 is a recombinant of TM-1 and national check variety 'Varuna'.

In recent years, a large number of morphological mutants, including yellow seed coat mutants, have been isolated from different varieties. Quality improvement in mustard is related to reduction of erucic acid (normally 45-50%) in oil and of glucosinolates (normally>150 µmoles/g) in oil free meal. As both

traits are nutritionally undesirable, it is important to develop zero erucic acid and zero glucosinolate selections called double zero (00) lines. Such double zero lines were generated. However, there appears to be yield penalty for these lines compared to normal varieties. Efforts are being made to develop high yielding "double zero" lines.

## Protein Staples

### Trombay pulses

Crop improvement programme in pulses was initiated during the early 1970's in three important crops namely pigeonpea, mungbean and blackgram for induction of mutations for yield, quality, disease and pest resistance. Later soybean and cowpea were added to the list. A large number of mutants with altered characteristics were isolated and used in genetic analysis and cross-breeding programme. Sustained efforts for development of pulse varieties resulted in the release, for commercial cultivation, of eleven varieties, four of mungbean, four of blackgram, two of pigeonpea and one of soybean.

Diseases and infestation with pests are major constraints in realizing high yield potential of pulse crops. Powdery mildew and yellow mosaic virus (YMV) diseases of mungbean and blackgram, *Fusarium* wilt and sterility mosaic diseases of pigeonpea and bacterial leaf pustule disease of soybean are the most damaging diseases. Screening techniques for identification of pest-resistant genotypes among radiation-induced mutants were developed for mungbean, pigeonpea and soybean.

In pigeonpea, several wilt resistant selections were further developed and are at various stages of testing at state and national levels. Efforts on breeding for resistance to powdery mildew disease resulted in development of three mungbean varieties, TARM-1, TARM-2 and TARM-18. Genetic stocks of blackgram with combined resistance to powdery mildew and YMV were developed.

Bruchid (*Callosobruchus maculatus*) is a serious store-grain pest of pulses. Few varieties are resistant to bruchid. A wild progenitor of blackgram, *Vigna mungo* var *sylvestris* collected from Trombay hills was found to be resistant to bruchid damage. The resistance has now been transferred to the elite background of blackgram variety TU-94-2.

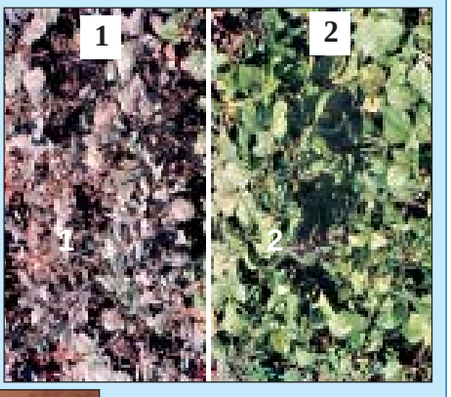
**Trombay groundnut varieties released and notified for commercial cultivation**

| Name/ Year of Release | Released for | Characteristics Maturity (M) days and Yield (Y) kg/ha |
|---|---|---|
| TG-1<br>1973 | Maharashtra,<br>Gujarat | M: 130-135<br>Y: 2400-2500 |
| TG-17<br>1985 | Maharashtra | M: 115-120<br>Y: 1400-2000 |
| TG-3<br>1987 | Kerala | M: 110<br>Y: 2000-2500 |
| Somnath<br>(TGS-1)<br>1989 | Gujarat | M: 110-115<br>Y: Kharif 2000 |
| TAG-24<br>1992 | NATIONAL CHECK VARIETY<br>Maharashtra,<br>Karnataka,<br>West Bengal,<br>Rajasthan | M: Kharif: 100-105<br>Summer: 112-117<br>Y: Kharif: 1300<br>Summer: 2500 |
| TG-22<br>1992 | Bihar | M: Kharif:115-120<br>Y: Kharif: 1677 |
| TKG-19A<br>1994 | Maharashtra | M: 120-125<br>Y: Summer 2000-2500 |
| TG-26<br>1995 | Gujarat,<br>Maharashtra,<br>Madhya Pradesh | M: 110-115<br>Y: Summer 2500 |
| TPG-41<br>2004 | All India | M: 115-120 days<br>Y: Summer 2338 |
| TG-37A<br>2004 | Rajasthan,<br>Punjab,<br>Haryana,<br>Uttar Pradesh | M: 110-115<br>Y: Kharif 1993 |

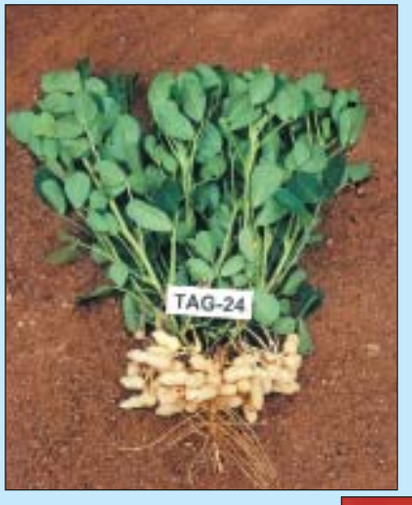
## Trombay pulse varieties released and notified for commercial cultivation

| Name/ Year of Release | Released for | Characteristics Maturity (M) days and Yield (Y) kg/ha |
|---|---|---|
| Mungbean TAP-7 1983 | Maharashtra, Karnataka | M: 60 Y: 700-800 |
| TARM-2 1992 | Maharashtra | M: 90 (Rabi) Y: 1000-1100 |
| TARM-1 1995 | Maharashtra, Gujarat, Madhya Pradesh, Andhra Pradesh, Karnataka, Kerala, Tamil Nadu, Orissa | M: 80 Y: 765 |
| TARM-18 1995 | Maharashtra | M: 65-70 Y: 1051 |
| Blackgram TAU-1 1985 | Maharashtra, Karnataka | M: 70-75 Y: 800-1000 |
| TAU-2 1992 | Maharashtra | M: 70 Y: 900-1000 |
| TPU-4 1992 | Maharashtra, Madhya Pradesh. | M: 70-75 Y: 900-1000 |
| TU-94-2 1999 | Andhra Pradesh, Karnataka, Kerala, Tamil Nadu | M: 70 Y: 900-1000 |
| Pigeonpea TT-6 (Trombay Vishakha-1) 1983 | Madhya Pradesh, Maharashtra, Gujarat, Andhra Pradesh, Karnataka, Kerala | M: 135-140 Y: 1200-1300 |
| TAT-10 1985 | Maharashtra | M: 110-115 Y: 900-1000 |
| Soybean TAMS-38 2004 | Maharashtra | M: 90-95 Y: ~ 2000 |

*Trombay Akola Rabi Mung (TARM –1)*




*Powdery mildew sensitive (1) and resistant mungbean(2)*




*Trombay Akola Groundnut (TAG–24)*

*Trombay Sesbania rostrata-1(TSR-1)*

## Quality improvement of wheat

Wheat is an interesting plant because of its genetic complexity and economic importance. Although wheat grain contains lower protein percentage than legume grains, its regular inclusion in sizable amount in diet makes it an important source of protein. Keeping in mind the need to improve nutritional quality of consumers there was considerable interest in improvement of wheat grain protein percentage. Basic studies in biochemistry and physiology of nitrogen utilization by the plant and its conversion into grain protein were carried out using contemporary techniques including use of radioisotopes. These revealed that grain protein content was a complex trait and that it had a negative association with grain yield. With increased availability of wheat to consumers, thanks to the green revolution. the emphasis changed from study of nutritional quality to study of role of proteins in processing quality. Wheat varieties with lower protein content and weak gluten were more appropriate for biscuit making while bread making quality was associated with higher protein content and stronger gluten. It was also known that a small fraction of the total protein was responsible for major part of the differences in the gluten strength. This fraction contained high molecular weight subunits of glutenin. A hypothesis was proposed that if a particular subunit was good and its genetic alternative (encoded by an allelic gene) was poor, it should be possible to substitute one for the other and the change in the quality should be observed. In a back crossing experiment a poor quality subunit was substituted by a good quality subunit in a common wheat variety "Kalyan Sona". The substitution resulted in improvement in bread making quality.

In a crossing programme appropriate recombinants were obtained which demonstrated that improved bread making quality could be obtained in combination with moderate rust resistance without loss of yield potential. Traits like rust resistance and yield were not easy to work with in the field experiments because expression of the contributing genes was easily altered by environmental conditions. Marker assisted selection technique offers a suitable alternative.

## Trombay *Sesbania*

*Sesbania* species are promising nitrogen fixing legumes. *Sesbania* offers potential for farmers to grow their own nitrogen instead of depending on chemical fertilizers alone.

Unlike other green manure plants, some *Sesbania* species have nitrogen-fixing nodules on their stems and roots. *Sesbania* has 5 to 10 times more nodules than most legumes. Since *Sesbania* can be ploughed back in 45 to 60 days, it can fit between two rice crops. Initial experiments showed that in about 55-60 days the *Sesbania* crop fixed about 120 kg nitrogen per hectare. *Sesbania rostrata* showed tolerance to salinity, acidic and alkaline soils.

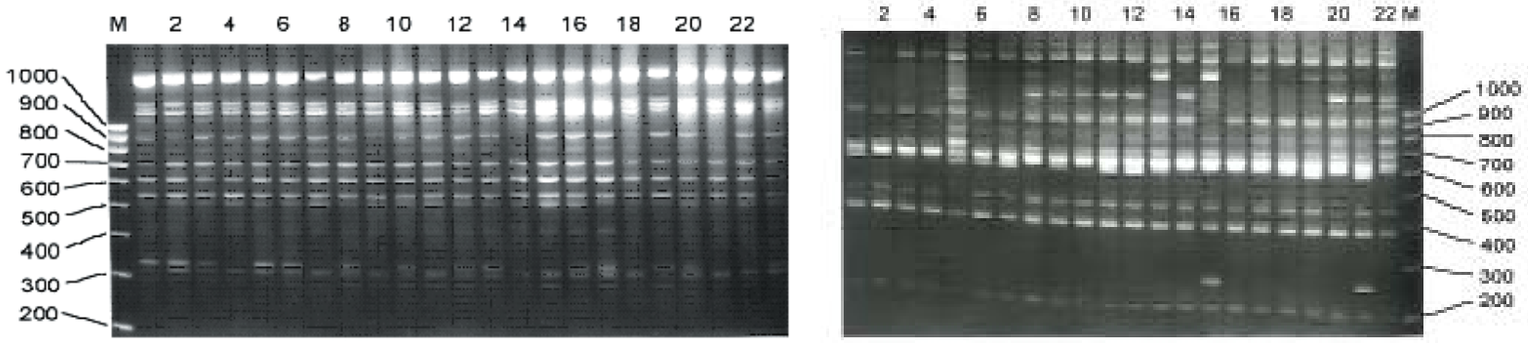
A late flowering mutant named Trombay *Sesbania rostrata-1* (TSR-1) was isolated which was insensitive to photoperiod and produced in *rabi* season vegetative growth for about 70 days while the parent produced vegetative growth for only about 35 days.

## Keeping up with the technological advances

The first generation of mutant cultivars described above was developed following largely the classical methods of mutation induction and selection. In order to take these mutant cultivars to the farmers, new programmes were initiated in the 1980's. These included search for mutations determining host-pathogen and host-insect pest interactions, anti-nutritional factors such as trypsin inhibitors, and proteins associated with superior grain quality. Earlier research on genetic polymorphism based on protein and isozyme markers was extended using DNA markers based on Randomly Amplified Polymorphic DNA (RAPD). These techniques led to development of molecular methods for varietal identification, checking purity of hybrid seeds, tagging of mutant genes and marker-assisted selection. DNA-based molecular markers have shown promising results in cotton hybrids for testing the genetic purity of hybrid seeds.

Blackgram genotypes and varieties developed at Trombay were characterized using different molecular marker techniques namely, RAPD, ISSR (Inter Simple Sequence Repeat) and AFLP (Amplified Fragment Length Polymorphism). Molecular markers were developed for detection of rice blast pathogen *Magnaporthe grisea* which is one of the main pathogenic threats to rice crop worldwide. A diagnostic assay based on the polymerase chain reaction was developed for detection of seeds infested with the blast fungus.

Availability of DNA markers has resulted in initiation of genome mapping programmes in different living organisms. Utilization and maintenance of a germplasm is greatly benefited

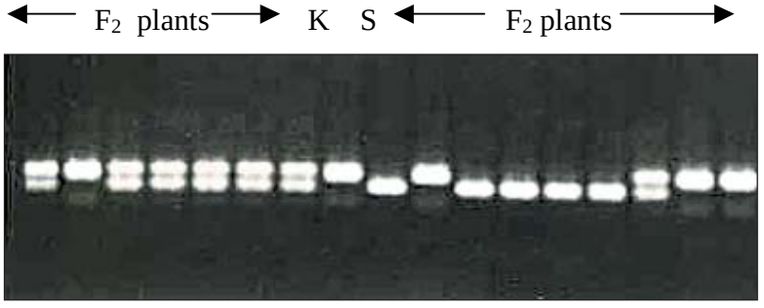



*ISSR molecular markers for mungbean. Each lane corresponds to a different genotype*

### Special long primers to study genetic diversity

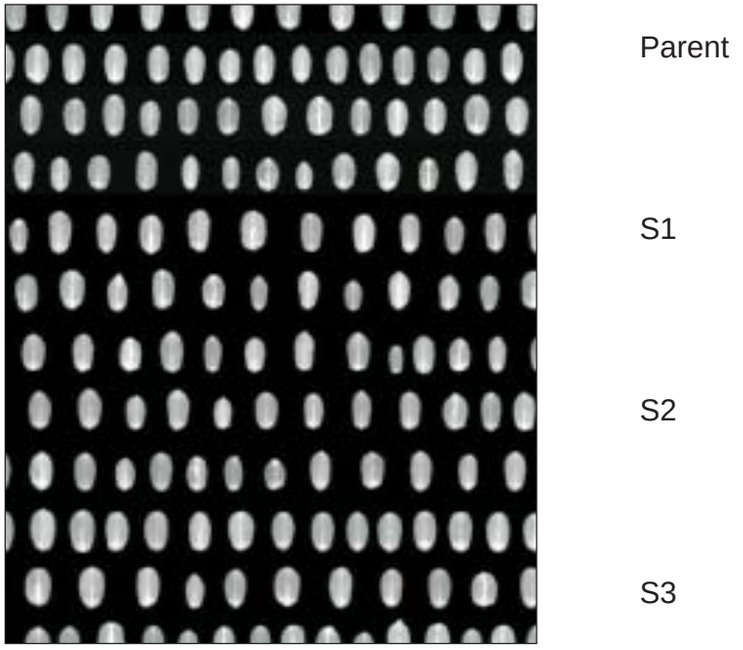
Using a set of long primers the genetic diversity among 48 wheat varieties was analyzed. By carrying out the association analysis, markers associated with the 1B/1R translocation in wheat were identified. The translocated fragment from rye chromosome 1R has served as a source for a number of useful genes including rust resistance genes.

by the availability of easier and accurate markers for identification of the genotypes. DNA markers were developed for this purpose for mungbean, groundnut and wheat. In case of mungbean ISSR markers were found to be highly informative, a profile obtained from two primers being sufficient for identifying all 100 genotypes included in the study. Among molecular markers the most informative are the DNA markers for 18S, 5.8S and 26S ribosomal RNA genes along with an Internal Transcribed Spacer (ITS) and an Intergenic Spacer (IGS) region. These markers are proving very useful for deciphering evolutionary relations among different genotypes, especially of mungbean.




*Segregation of Simple Sequence Repeats (SSR) marker in chromosome 4B in $F_2$ plants derived from a cross between two wheat varieties Sonalika (S) and Kalyansona (K)*

### Computer vision techniques for morphometric characterization of wheat grains




*Distinguishing genetically close selections in wheat using computer vision techniques. Grain images of parent variety and three selections (S1, S2 and S3)*

Visual observation of the shapes and sizes of grain is the usual way to identify a variety. This, however, requires expertise. Computer based image analysis could provide an objective way of varietal identification. Comprehensive Image Processing Software (CIPS) has been developed in Computer Division, BARC for morphometric analysis of wheat grains. Gray images of the wheat grains are captured. The software detects each grain in the image and rotates for normalization of orientation. These rotated images are used for quantitative

measurement of 45 shape and size related parameters. The data for each parameter for every grain in the image as well as mean, standard deviation and standard error for that parameter are stored for further use. Using this imaging system shape variation, based on grain morphology was quantified in fifteen Indian wheat varieties, as well as among the genetically related wheat selections. Euclidean distances calculated using differences in means could serve as a basis of distinguishing between varieties and genetically related samples.

## Basic Studies

Bread wheat is hexaploid, which means there are six copies of each gene. That makes it tolerant to loss of an arm of a chromosome or an entire chromosome. Using such cytogenetic stocks called monosomics, location of certain genes was found out and the genes were also assigned positions on the chromosome arms. Combination of electrophoretic and cytogenetic techniques allowed study of isozymes (which are variant forms of an enzyme) and their chromosomal location. Isozymes of alcohol dehydrogenase (ADH) were studied. A variant form of this enzyme was detected in tetraploid wheat and then transferred to hexaploid background. The monosomic series available in wheat variety "Chinese Spring" did not serve the purpose all the time. It was thought that a series developed in a local variety would be helpful. Some monosomics were obtained in the background of cultivar Kalyansona for cytogenetic studies. Variability in morphological characters, storage proteins and isozymes of wheat was limited. However, much greater variability was expected to be present at the DNA level. Variation in the ITS region was detected in the hexaploid cultivars and one such variant was mapped on to the respective chromosome.

Re-association rate studies of dissociated DNAs from diploid, tetraploid and hexaploid wheat species revealed that all species had a large proportion of their DNA as repetitive DNA. The genome of wheat species contains about 70-89% of the total DNA as repetitive DNA. The amount of non-repeating DNA was large in hexaploid and tetraploid wheat in comparison with diploid wheat. Heterologous re-association of repeating and non-repeating fractions of DNA showed considerably more divergence in the repeated sequences at both diploid and polyploid levels. Non-repeated sequences of wheat species showed greater homology and appear to be more conserved in composition.

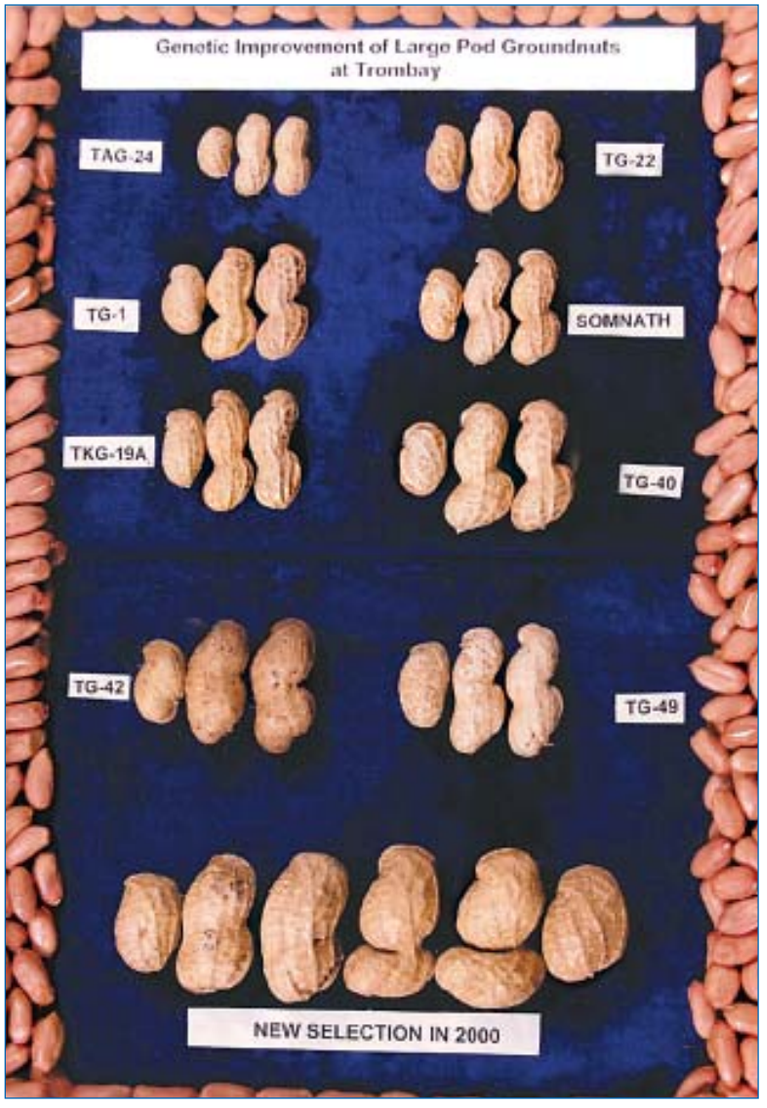
The structural genes of NAD-dependent ADH isozymes are located on chromosome 4A and 4B. The *Rht* gene responsible for reduction in plant height is also located on chromosome 4A. Studies were initiated to detect the linkage between *AdhA1* gene and *Gai1/Rht1* gene and to map the *AdhA1* gene. *AdhA1* gene was estimated to be 23 crossover units from *Gai1/Rht1* gene, which was 15 crossover units from centromere, showing a linkage between the two genes. ADH isozymes of mutant type were purified and characterized. The results indicated that the variant isozyme is the result of a mutation altering only the charge of the isozyme.

Studies on genetics of resistance to diseases and pests of pulse crops revealed that resistance to *Fusarium* wilt in pigeonpea was governed by a single dominant gene while two dominant genes controlled the powdery mildew resistance in mungbean. While duplicate recessive genes controlled the resistance to bacterial leaf pustule disease in soybean, two dominant duplicate genes determined the resistance to bruchid in *V. mungo* var sylvestris.

*Improved pulse varieties developed at Bhabha Atomic Research Centre, Trombay*

## Trombay Rice Variety "HARI"

- "Hari" a high yeilding semi-dwarf rice variety developed by BARC in collaboration with Andhra Pradesh Agriculture University and the Department of Agriculture, A.P.
- Notified by Ministry of Agriculture, Govt. of India, in the year 1988.
- "Hari" evolved from the cross IR-8 x TR-5. (a fast neutron induced dwarf mutant of SR-26B).
- Semi-dwarf, erect, compact and non-lodging plant type.
- Duration : 135-145 days in Kharif.
- Grain : Long slender, straw-yellow colour .
- Kernel : White, flinty, translucent and non-glutinous, white belly absent; good cooking quality.
- Optimum sowing time : Kharif - early June, Rabi - mid December.
- Yield : 5939 kg/ha; 20% higher over check variety.
- "Hari" not affected by green leaf hopper, leaf folder pests, blast, tungro virus, sheath blight and brown leaf spot diseases.



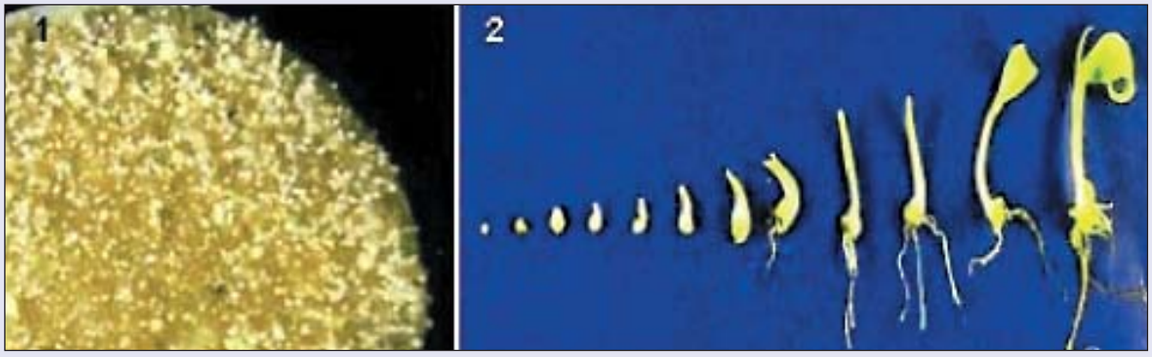

*Lush-green fields of TAG-24*

# Plant Biology

## Link B2

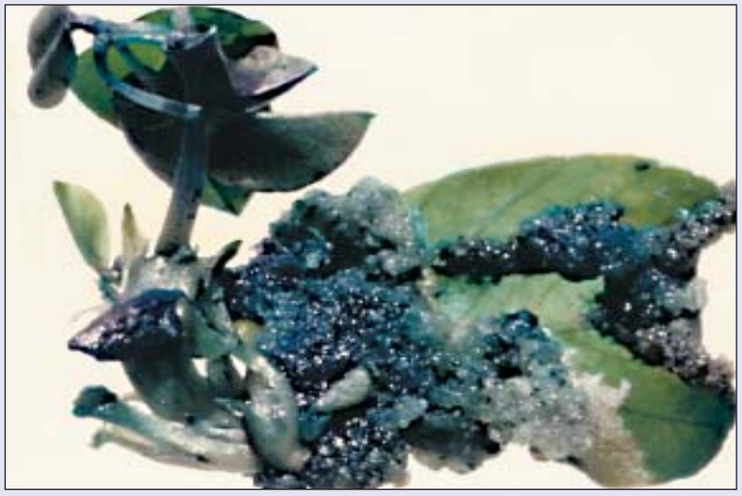
*Plants stand at the interface of inorganic and organic world. The mind-boggling mystery of how plants, and other aquatic and terrestrial autotrophs, use energy from visible light to sustain life on earth has kept generations of researchers occupied. Plant biology has taken tremendous strides in the last fifty years. Plants constitute unique systems to decipher genetic basis of physiological and biochemical phenomena. Plant breeders are the most successful genetic engineers. They can shuffle genes by making intelligent choice of parents in breeding, a distant dream in human biology. The success story of plant breeding has resulted in making India self-sufficient in food. Many plants show totipotency, that is ability of a single somatic cell to give rise to an entire plant. This character was harnessed in development of plant tissue culture to produce micropropagated plants in vitro as well as synthetic seeds. Plants are sources not only of food and nutients but also of several commercially produced drugs and formulations. With the advent of recombinant DNA techniques several genes of microbes, plants, animals and even humans can be expressed in plants to enhance their value as crops or for providing value added products. Bioenergetic constraints on increase in yield, protein and oil content of crop plants has generated interest in basic research in different areas of plant biology such as plant physiology, photosynthesis, nitrogen assimilation, mineral nutrition, plant pathology etc. Understanding basic mechanisms of the underlying biochemical processes in plants has proved to be a very challenging task.*

# Plant tissue culture




*Somatic embryos of banana (1), Stages during the conversion of somatic embryos into banana plants (2)*

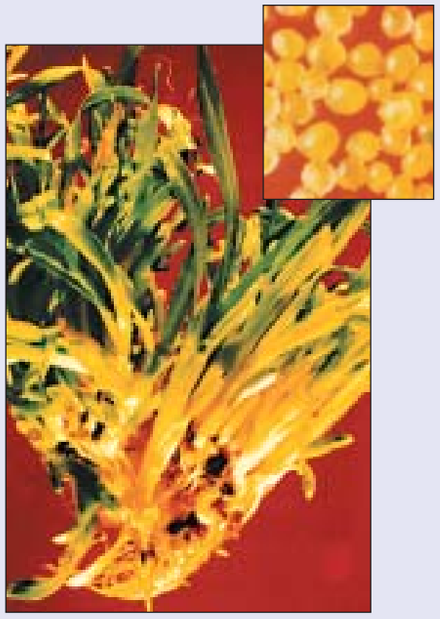
*Somaclonal variation in finger millet induced in tissue culture*



*A transgenic groundnut plant expressing Gus activity*

*Pineapple (inset) developed by tissue culture technique*

*Haploid plants of Brassica generated from pollen (inset)*



*Finger millet plants from synthetic seeds (inset)*

Radiation had found a great use in generation of mutants of crop plants. A careful study showed that radio-sensitivity of maize and barley seeds varied according to their moisture content. Also inbred lines compared to hybrids in maize showed differences in radiosensitivity. Ionizing radiation caused stunting of maize seedlings, which could be reversed by gibberellic acid (a plant hormone). These early observations indicated a complex relationship between physiological state of the plant, DNA damage manifestation and dose of ionizing radiation. Frontiers of research in plant biology thus slowly shifted from plant breeding and cytology to understanding basic biology of plant systems. Over the years motivated young people in BARC aided by continuous introduction of new facilities generated a great deal of expertise in plant biochemistry, characterization of mutant genes, DNA profiling, gene cloning and development of transgenics. Active areas of basic research in plant biology have been variability in seed proteins, isozyme markers, pathogen resistance, enzymes of photosynthesis and nitrogen assimilation and their regulation, plant tissue culture, plant growth regulation, soil science and fertilizers, plant protection from insect pests and pesticide degradation etc. With active multidisciplinary collaboration the first genetically engineered plant in India was produced at BARC, just three years after first such reports from Europe and USA.

## Seed proteins

Biochemical and genetic characterization of seed storage proteins of wheat, pigeonpea, mungbean, blackgram and groundnut was carried out. Likewise, inheritance of charge variation in vicilin of mungbean was investigated. A globulin fraction rich in sulphur aminoacids was discovered in pigeonpea. At least four genes were found to be responsible for coding constituent polypeptide components of arachin, a major seed protein of groundnut. Two of these genes were non-allelic. The non-allelism led to expression of four polymorphic forms of arachin found in groundnut.

Two protease inhibitors were purified from pigeonpea seeds. One of them inhibited both trypsin and chymotrypsin while the other inhibited only trypsin. The purified inhibitors in solution were stable at $80^0$ C for 15 min and pH 7-10. The inhibitors were very specific towards mammalian serine proteases. They did not inhibit other proteases or serine proteases of bacterial origin. During germination, the inhibitor activity decreased from six days after germination up to ten days.

## Bioenergetics of plant productivity

The main focus in plant breeding was to isolate mutants with higher yields. A consistent negative correlation was observed in different crops between (a) number and mass of grains, (b) grain yield and grain protein concentration, (c) protein and oil concentration in seed, and (d) oil concentration and yield. It was difficult to visualize a genetic basis for such negative association. It suggested that there was perhaps competition for the product of photosynthesis (glucose). A theoretical calculation of bioenergetic costs of oils from different oil-seed crops based on their standard fatty acid composition and seed biomass showed that increase in oil percentage in seeds had maximum energy cost. This showed that there could be bioenergetic constraints for improving quantity or quality of protein or oil with simultaneous increase in grain yield, unless total energy input in terms of photosynthate was enhanced. Similarly, genetic resistance to disease and pests had bioenergetic costs.

## Photosynthesis

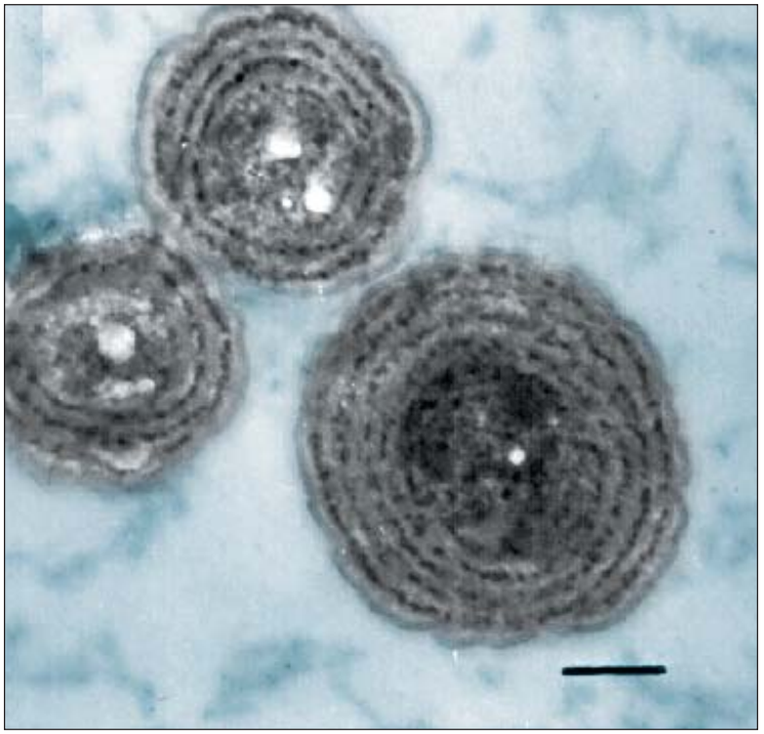
Photosynthesis is a process that converts solar energy into chemical forms as compounds with high energy and fixes atmospheric $CO_2$ to produce food and fibre. Research in light and dark reactions of photosynthesis was initiated in 1971.

Mechanism of photosynthetic electron transport was deciphered employing the technique of thermoluminescence (TL). Using an indigenously built apparatus several important findings relating glow curves to back reactions in photosystem II (PSII), delayed light emission from photosystem I (PSI), involvement of “S” states of water oxidation complex in TL and relationship of TL with decay component in delayed light were reported. Seven distinct glow peaks were observed which were related to various steps in electron transport using inhibitors.

Progenitors of cyanobacteria faced far-UV solar radiation two billion years ago when ozone protection shield was very

thin; hence they may have evolved novel repair systems. Unlike other bacterial cells preincubation of cyanobacteria in light reduced their survival after UV exposure. It was concluded that PSII was likely to be a lethal target of far-UV radiation. This was confirmed using UV resistant and DCMU resistant mutants.

Several plant enzymes involved in carbon assimilation and nitrogen metabolism were purified and analyzed for structure-function relationships, mechanism of action and regulation; many of them for the first time in the country. Isolation and purification of plant enzymes was difficult due to presence of several interfering compounds in plant system, and also due to differential expression of plant enzymes in response to environmental conditions.



*Transmission electron micrograph of Anacystis nidulans showing protein crowded environment around thylakoid membranes in vivo. (Magnification 30000, Bar-200 nm)*

In carbon assimilation Calvin cycle is the principal pathway. RuBP carboxylase-oxygenase (Rubisco) is responsible for assimilation of atmospheric $CO_2$. Active site mapping studies were done using selective chemical agents binding to specific amino acids, fluorescence and affinity labels. These studies showed a role for histidine, tryptophan, tyrosine and lysine residues in the active site of Rubisco. Proximity of histidine to active site lysine and cysteine residues was also clearly demonstrated using affinity labels. Activation by bicarbonate and magnesium is a prerequisite in catalysis by Rubisco. An elegant study using a fluroescence probe indicated that the pH at the catalytic site of unactivated enzyme was highly acidic (pH<2) and activation by bicarbonate and magnesium resulted in a conformational change in the enzyme molecule so that the active site became highly polar and basic in nature.

Traditionally, all the soluble enzymes are studied *in vitro* in isolation. However, these enzymes function precisely *in vivo* in an environment crowded with proteins and limited by water. Such ambience will necessitate that sequential enzymes of metabolic pathways have to be spatially organized. Multienzyme organization among sequential enzymes of Calvin cycle was, therefore, investigated. Immunogold cryoelectron microscopy provided evidence that Calvin cycle enzymes might be organized as a thin layer on the surface of thylakoid membranes. Membrane-associated enzyme assembly accounted for an efficient coupling between photosynthetic electron transport and $CO_2$ fixation.

Wild relatives of wheat have higher rate of photosynthesis per unit leaf area compared to the cultivated hexaploid wheat. The morphological-anatomical differences between the two were thought to be a contributing factor. Wild relatives have narrow leaves with higher stomata per square millimeter of leaf surface. Bread wheat, in contrast, has wider leaves and lower stomatal frequency. Influence of stomatal frequency on the photosynthetic rate was evaluated. The study revealed that stomatal frequency had a genetic component and the trait was heritable. Some of the selections had higher oxygen evolution rate indicating higher photosynthetic potential.

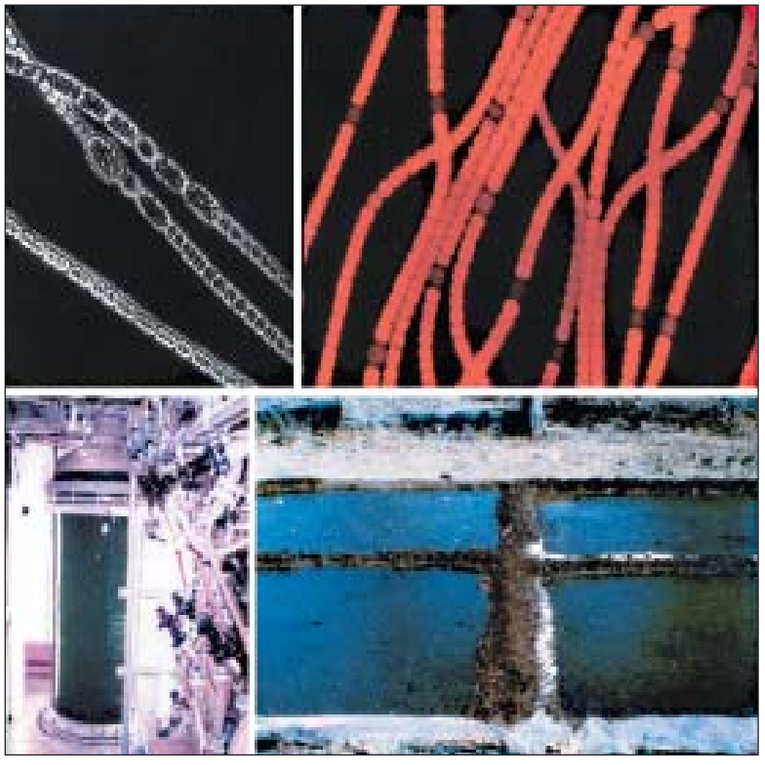
### Biological nitrogen fixation and nitrogen assimilation

Nitrogen is important and usually considered a limiting factor for growth of plants. Assimilation of nitrogen requires a complex series of biochemical reactions, which are the most energy requiring reactions. Plants such as legumes form a symbiotic relationship with nitrogen fixing bacteria to convert molecular nitrogen into ammonia. Alternatively, plants can assimilate nitrate into ammonia and finally into the aminoacids. Assimilation of nitrogen in proteins in photoautotrophs was an important area of research in BARC.

Cyanobacteria, also called blue green algae, are obligate, oxygenic photoautotrophs and some of the strains with heterocysts can fix atmospheric nitrogen. In filamentous cyanobacterial strains *Anabaena* L-31 and *Nostoc* differentiation into heterocysts was found to be more frequent under nitrogen-limiting conditions. Using an elegant microspectrophotometric technique, heterocysts of *Anabaena* were shown to lack the oxygen-evolving components of photosystem-II (phycocyanin) and possess a highly reducing intracellular microaerophilic environment, conducive for nitrogenase function. These studies, provided the first *in situ* evidence for heterocysts being the sites of aerobic nitrogen fixation. Pioneering work, using a short-lived isotope of nitrogen, $^{13}N$, later confirmed the role of heterocysts as sites of nitrogen fixation and ammonia assimilation in *Anabaena*. A peptide inducing heterocyst differentiation and another inducing sporulation in *Anabaena* were also reported. Symbiotic relationship between vegetative cells and heterocysts was observed in that nutrients were supplied from vegetative cells to heterocysts and fixed. Nitrogen was exported from heterocysts to vegetative cells. Continuous cultivation of filamentous cyanobacteria was studied. By adjusting the flow rate of nutrients and products it was possible to achieve continuous $N_2$ fixation in the fermenter vessel even in the presence of $NO_3^-$ as the nitrogen source. Mass cultivation of nitrogen fixing cyanobacteria and their application as biofertilizer in rice fields was studied. *Nostoc*-4 emerged as the best strain, which could withstand high temperature, high light intensity and still fix high amount of $N_2$. Nitrogenous fertilizer requirement could be supplemented by using this biofertilizer.

A novel but specific requirement for a trace quantity of sodium for cyanobacterial growth, photosynthesis and nitrogen fixation was discovered and the molecular basis of pleiotropic effects observed in cyanobacteria under sodium deprivation was investigated. Using EPR spectroscopy sodium starvation was shown to result in an inactive MoFe protein with an altered active site in *Anabaena* heterocysts.

Subsequently, studies on the genetic regulation of nitrogen fixation were actively pursued. Early events in the establishment of legume-rhizobium symbiosis were studied in cowpea system. This study showed presence of dual binding sites for peanut lectin on rhizobial cell surface. Production of root exudates by legume host triggered nodulation-related events in the rhizobial partner. Some factors responsible for specificity and competence in legume-rhizobium symbiosis were identified.



*Nitrogen fixing stress tolerant strains of cyanobacteria: Clockwise from top: filaments with spores and heterocysts; fluorescing vegetative cells and non-fluorescing heterocysts; cyanobacteria growing in paddy-fields at Trombay; and 1500L fermentor culture of Anabaena sp. strain L-31*

The other important source of nitrogen for plants is through assimilation of nitrate by nitrate reductase. Investigations on the structure and function of plant nitrate reductase (NR) led to several interesting findings regarding partial activities of this enzyme. Activity of nitrite reductase results in formation of ammonia from nitrite by using light energy. In plants ammonia is mainly assimilated into glutamate by glutamine synthase and glutamate synthase. Glutamine synthase (GS) plays a key role in nitrogen metabolism of microbes and plants. GS was purified to homogeneity from *Anabaena* L-31 and from rice plant leaves and roots and its regulation was extensively studied.

The amino group from an aminoacid is transferred to a keto acid by the transaminases or aminotransferases. Among the aminotrasferases aspartate aminotransferase from mungbean

was extensively investigated. Two isozymes of aspartate aminotransferase were characterized. The other enzymes in biosynthesis of aspartate family aminoacids such as aspartokinase and homoserine dehydrogenase were also studied. These enzymes are under feedback control of lysine and threonine. Based on this, a simple procedure to screen lysine+threonine resistant mutants was devised.

### Soil science and fertilizers

Both natural and artificially produced radionuclides are useful to study efficiency of different fertilizers for various crops and uptake, translocation and localization of various elements in plants. Initial investigations on plants grown in high natural radiation area of Kerala coast and adjoining regions having monazite bearing soils clearly established uptake of natural radionuclides of thorium series ($^{228}$Th, $^{224}$Ra, $^{228}$Ac, $^{212}$Bi) by plants. Later, the uptake, distribution and metabolic fate of long-lived gamma-emitting fission products like $^{137}$Cs, $^{125}$Sb, $^{106}$Ru, $^{144}$Ce and gamma-emitting activation products such as $^{59}$Fe, $^{58}$Co, $^{54}$Mn, $^{65}$Zn were evaluated in plants like maize, bean, etc. grown in hydroponics as well as in different Indian soil types. In general, results indicated massive accumulation of these radionuclides in roots and little uptake by shoots. In the shoot, most of the radionuclides followed an acropetal gradient. Soil amendments like organic matter and lime addition reduced the movement, whereas, synthetic chelates enhanced the mobility of these radionuclides. In general, the uptake of these radionuclides was found to be higher from acidic soils than from alkaline soils.

Studies on the efficiency of different phosphate fertilizers such as ammonium nitrate phosphate (ANP) and ammonium polyphosphate (APP) for crops on Indian soils led to development of a new product ANP (20-20-0) with 60% water soluble phosphorus (WSP), which was then produced commercially by Rashtriya Chemicals and Fertilizers Ltd. (RCF), Mumbai to be used for neutral and alkaline soils.

Uptake of toxic heavy metal pollutants like Cd by plants, from different soil types, sewage sludge, city composts – amended soils and rock phosphates applied soils was evaluated. In general, the adsorption capacities of soils for Cd increased with increase in pH. Synthetic chelates effectively enhanced the mobility of Cr and Cd.

## Plant Molecular Biology and Biotechnology

### Plant tissue culture

Experiments on plant morphogenesis and tissue culture were initiated in 1964. Later, the focus changed from mere morphogenesis to Plant Biotechnology. Culturing of anthers of *Datura* species led to generation of not only haploid plants but also diploids and triploids. Based on these findings the origin of triploids was proposed to be due to fusion of vegetative and generative nuclei of microspore, which was confirmed by other scientists. Later, haploid plants were generated in *Atropa belladonna, Capsicum annuum* and *Brassica juncea.* Methods for protoplast culture of *Arachis hypogaea, Santalum album, Physalis minima, Tylophora indica, Pergularia pallida,* and *Morus indica* were developed. Successful plantlet regeneration from protoplast was obtained in *Brassica juncea, Santalum album*, *Vigna aconitifolia* and *Tylophora indica*.

Studies on cells, organ and callus cultures were conducted in many plants. Plant regeneration through organogenesis/ somatic embryogenesis/multiple shoots was obtained in several cereals, pulses and medicinal plants. However, several somaclonal variations were observed in plants regenerated in tissue culture. An interesting example is the isolation of a yellow seeded somaclonal variant of mustard from black seeded *Brassica juncea*.

Micropropagation techniques for rapid propagation of Sandalwood, Mulberry, Banana, Pineapple, Grape, Potato, Cardamom and Sugarcane plants were established. Experiments for high conversion of somatic embryos to plants were carried out in sandalwood, banana and sugarcane. Banana micropropagation protocol was given to user agencies for commercial production.

*In vitro* culture methods are being used for production of several bioactive compounds from *Rauwolfia serpentina, Atropa belladonna, Tylophora, Catharanthus roseus, Nothapodytes foetida*, *Azadirachta indica, Hemidesmus, Castanospermum australe and Coleus forskolhi.* The notable examples of bioactive compounds are camptothecin from *Ophiorrhiza* cultures, 5-MPT from *Linum flavum* root cultures and ajmalicine from *C. roseus*.

## Bioreactor technology

*Bioreactor (100 L) equipped with helix impeller for growth of Catharanthus roseus cultures*

Bioreactor provides nutritional and closely controlled environment for optimum growth of plant cells in which cells perform biochemical transformation to synthesize bioactive compounds. *Catharanthus roseus* (*Vinca rosea*) cell cultures were grown in a 100 L bioreactor equipped with an helix impeller. An 11.5% inoculum yielded a 32 kg fresh biomass within 16 days of cultivation, whereas naturally grown plants produce 2.3 kg/$M^2$/year. Suspension cultures of *C. roseus* were cultivated in a 20 L air lift bioreactor and ajmalicine (yield 0.0135%) was obtained on day 15.

### Genetic engineering

The first genetically engineered tobacco plants expressing bacterial kanamycin resistance gene were produced in the country in 1986 in BARC. Inheritance of the transferred gene was also reported. Genetic transformation with *cry1Ac* and/or *nptII* genes was achieved for crop plants such as *Brassica juncea*, *Vigna aconitifolia,* groundnut, chickpea, mothbean, tobacco etc using various methods for gene transfer such as *Agrobacterium* mediated and/or direct DNA transfer (PEG mediated/electroporation/particle gun bombardment). Magainin, an antimicrobial peptide gene, was incorporated into Banana, Tobacco, Potato and Pineapple. Transformed tobacco plants showed resistance against the fungi *Sclerotia*, *Alternaria* and *Botrytis* fungus whereas transgenic banana plants were resistant to *Fusarium* and *Mycosphaerella*.

Coat protein gene from Potato Virus Y was cloned and incorporated into tobacco and potato for resistance against the virus PVY. Confirmation that the transgenic plants had acquired resistance came from bioassay performed at Central Potato Research Institute, Shimla. Currently efforts are on for genetic transformation of banana plants using a variety of genes such as replicase gene of Banana bunchy top virus, anti-sense ACC synthase gene and hepatitis B surface antigen coding gene.

### Sterile insect technique

Radiation adversely affects the reproductive system and makes insects sterile. Taking advantage of this fact, E. F. Knipling in 1930 conceived the idea of controlling, managing or eradicating insect pests from a defined area by affecting reproductive capacity of the insects. Continuous release of a large number of mass produced sterile insects into natural population would limit the reproductive ability of the latter, which would result in reduction of density of natural insect population gradually leading to a possible elimination of pests from that area.

Pilot scale field trial was conducted at Kayangulam, Kerala to demonstrate feasibility of the SIT to manage the red palm weevil. The trial was carried out in collaboration with Central Plantation Crop Research Institute (CPCRI) over an area of 320 hectares. Similarly, feasibility of SIT in controlling multiplication of potato tuber moth in storage was assessed in collaboration with Central Potato Research Institute (CPRI) at its regional research centre at Rajgurunagar near Pune, Maharashtra.

In nature a balance is known to exist between plant feeding insects and their natural enemies. These natural enemies are self-perpetuating in the field. However, in off-seasons sometimes parasites fail to perpetuate due to absence of required host stage. In this situation, the release of sterile females of the host insects will help to provide the host stage constantly in the field. This combined approach for control of potato tuber moth by use of egg parasite, *Trichogramma chiloni* and radiation sterilized females of potato tuber moth was evaluated. *T. chiloni* could be reared on eggs laid by radiation sterilized females of potato tuber moth without any adverse effect on its development and reproductive performance. Since insects are also susceptible to diseases caused by viruses, bacteria and fungi, these insect pathogens (bacteria and viruses) were isolated from indigenous sources. Realizing the great potential

*Pheromone traps*

### The Pheromone trap for insects

Potato tuber moth (PTM) is an internal feeder, which causes major economic damage to potatoes in field and storage. A biological phenomenon was exploited to develop a method for its control. The female moths were shown to release a sex pheromone, which attracts males. This pheromone is produced in a gland, located in the abdominal tip of female. The male receives the pheromone molecules through receptors on the antenna. An electroantennogram was fabricated in BARC to trace the response of antennae of male insects to pheromones. Pheromone could also attract and trap the males from long distances. A dry trap was fabricated in BARC (registered in the International Pest Information Directory). The phenomenon of attraction of males by the sex pheromone has been exploited by deploying pheromone traps in the field for pest control. When these traps were deployed in the field, the males were confused regarding the location of the female and were unable to mate, resulting in mating disruption and pest control. Similar techniques are being used for other pests e.g. the bollworm of cotton.

of *Bacillus thuringensis* (Bt) based biopesticide in the control of insect pests, intensive efforts were made to develop the technology for mass production of Bt based biopesticide in collaboration with Hindustan Antibiotics Ltd.

Garlic oil was found to have insecticidal properties due to di-allyl-disulphide and di-allyl-trisulphide. Several trials for mosquito control were undertaken in collaboration with the Bombay Municipal Corporation to demonstrate the feasibility of use of garlic oil and two synthetic compounds.

## Pesticide biodegradation

Pesticides were introduced into intensive agricultural systems several decades ago. Organic insecticides viz alkyl thiocyanates and fungicides viz dithiocarbamates were introduced in 1930. Introduction of DDT marked a revolution in the pesticide field in 1942. A large number of other synthetic organic pesticides followed. During this period risks involved in usage of chemical pesticides started surfacing. These were apparently the result of ecotoxicological effects following intensive use of DDT and other organochlorine pesticides.

Pesticide-persistence is an ecological hazard. Studies were, therefore, conducted in various ecosystems for evaluation of the impact of pesticide persistence. They involved use of simple laboratory systems like biometer flask to complex systems like field lysimeters. Such studies have been useful in predicting the possible bioaccumulation potential of either the parent molecule or its degradation products, in determining the waiting period for consumption of pesticides and in tracing the path of these chemicals in ground water.

A continuous flow system developed indigenously has helped in mass balance studies of several $^{14}$C-labelled pesticides like carbofuran, chlorpyrifos, oxyfluorfen, nitrofen, HCH, DDT, endosulfan etc. Using $^{14}$C-HCH and $^{14}$C-carbofuran. It was observed that there was no bioaccumulation of these pesticides in catfish and it took about a week after the application of these pesticides to reduce to zero level. Use of green manure to fields reduced the persistence of $^{14}$C-DDT, $^{14}$C-HCH, $^{14}$C-carbofuran and $^{14}$C-nitrofen especially under flooded conditions.

Studies on the behavior of $^{14}$C labeled degradation products of DDT revealed no residues in rice grains. The flooded soil conditions in rice fields and relatively higher temperatures further accentuated this degradation.

# Food Science

## Link B3

*There is a perpetual chase by agriculturists to provide enough food to keep pace with the increasing world population and yet food safety for the entire world remains a dream. The world population in the year 2000 was around 6 billion, which is likely to double in the next 50 years. Fifty percent of the world's land, that has agricultural potential, is in non agricultural use and this figure rises to around 90% in the highly populated areas of Europe and Asia. Most of the remaining area will be expensive to develop in financial terms, especially, when aridity and/or salinity are to be overcome. It will also be ecologically disastrous. Further, the existing area of cultivable land is decreasing due to urbanization, erosion, irrigation enforced salinity and overgrazing leading to desert formation. With the phenomenal increase in population and decrease in land potential, agricultural yield must increase over 200% between 2000 and 2050 just to maintain current levels of nutrition which are inadequate in many parts of the world. Considering the limited options for significant increase in food production, it is imperative that emphasis has to be laid on saving every grain of food produced. Thus food preservation is far more important in the present context of world food and population scenario. Food is processed for its preservation, hygienization or value addition. Traditionally, canning, appertization, using salts and sugar, drying and fumigation have been used to preserve food. Food irradiation is one of the newer techniques in food processing. Generally food irradiation implies use of ionizing radiations like gamma rays and electrons for controlling insect and microbial contamination and inhibiting certain physiological processes such as sprouting and ripening. Irradiation is a physical treatment and irradiated food is not a "processed food" in the classical sense, since irradiation does not change the identity of the food. For example freezing fruits would results in a change in texture and taste whereas irradiated fruits remain fresh. However, even with irradiation of foods "good manufacturing practices" and "good hygienic practices" are equally important.*

# Food preservation by radiation

*Fish & Meat (left), Spices (right). Irradiation improves shelf-life and hygienic quality of fish, meat, poultry and spices*

*Inhibition of sprouting in onion by radiation - processed (left) and unprocessed (right)*

*Inhibition of sprouting in potatoes by radiation - processed (left) and unprocessed (right)*

*Krushi Utpadan Sanrakshan Kendra, Lasalgaon (KRUSHAK)*

*Irradiated fruits and flowers*

Several thousand tons of food grains, fruit and vegetables, in warehouses and other storage places, are lost in India every year. The most important cause for this loss is inadequate post-harvest treatment and handling. It is paradoxical that food is enough yet cannot reach the needy. The importance of research and development in food science and technology was, therefore, amply stressed and addressed in the DAE programme on biological sciences right from its inception in the sixties with the establishment of a food irradiation and processing laboratory (FIPLY). Initial studies were directed towards determining the inhibitory effect of radiation on sprouting and its reversal by plant hormones. A major programme on irradiation of wheat was also undertaken. Several types of food materials such as grains, flours, vegetables, fruits, marine products, cut flowers and other products and irradiation processes, sometimes in combination with other hurdles, have been researched upon to investigate effectiveness of irradiation for their preservation. Based on the dose required for preservation, the applications are classified in three categories.

### Low dose applications

Studies on low dose applications (dose less than 1 kGy) formed the basis of commercial application of the technology for preservation of agricultural commodities. Inhibition of sprouting of onion, potatoes, garlic and ginger requires a dose of 0.05-1.05 kGy. Insect disinfestations of grains needs irradiation with 0.15 to 0.5 kGy. Delay in ripening can be achieved by irradiation with 0.5 to 1 kGy. A technology demonstration unit for low dose applications of radiation for preservation of agricultural produce, named KRUSHAK (Krushi Utpadan Sanrakshan Kendra) was set up by BARC at Lasalgaon near Nashik and it became operational in July, 2003.

Recent studies on mango, tomato, guava and amla demonstrated that a single radiation exposure of appropriate dose resulted in delay in the natural ripening process and senescence in climacteric and non–climacteric fruits, respectively. Film packaging further extended the advantages of irradiation by 10-15 days. Stem-end rot development in mango fruit greatly reduced its market value. Pre-irradiation dip treatment for 5 min in chitosan or turmeric solution was found to help in reducing the rot. Gamma irradiation at 1 kGy dose delayed the ripening of another climacteric fruit, green tomato. No significant differences in lycopene content, responsible for anti-cancer and anti-oxidant activities, were observed between irradiated and non-irradiated tomato. Gamma irradiation in the dose range of 0.1-0.5 kGy resulted in extension of shelf life of guavas. Storage at lower temperature showed maximum increase in shelf life as compared to that at ambient temperature. Ascorbic acid content of fruit remained unaffected following irradiation. Amla (*Emblica officinalis*), a tropical fruit with excellent nutraceutical properties and a rich source of vitamin C was found to be prone to fungal infection under anaerobic conditions created during bulk storage and conventional transportation. Gamma irradiation coupled with dip treatment in bavistin solution reduced the spoilage losses due to mold growth and senescence.

Extension in shelf life of more than one month was observed for flowers such as gerbera with gamma irradiation in combination with suitable packaging and storage conditions.

#### Food Irradiation

Food irradiation is a need-based technology used either for extension of shelf life or for improving safety of food. Generally, food irradiation implies use of ionizing radiations like gamma rays for controlling infestation with insects and microbes that causes food spoilage during storage. It also aims at prevention of physiological changes like sprouting and ripening in food. There are several advantages of radiation processing. It is a physical, non-additive process causing minimal changes in food. There is no perceptible change in organoleptic quality at the technologically recommended doses. Radiation used is highly penetrating and packaged products can be easily subjected to irradiation. It is a cold process and hence very effective for preservation of raw and frozen products. It is an excellent alternative to chemical fumigants and there are no toxic residues left in the food products making it a safe and environment-friendly process. Food irradiation has been applied for various purposes. The Governments of India and several other countries have approved a number of food items for preservation by irradiation.

### Clearance of irradiated food for human consumption

Safety of irradiated food for human consumption was always one of the major concerns for the commercial utilization of this new technology. Right from the first meeting organized by the International Atomic Energy Agency (IAEA), where 28 countries participated to review the progress in the field of food irradiation, health authorities in those countries were hesitant to grant permission for marketing irradiated foods for human consumption. Even at that time, three countries Canada, United States and the Soviet Union, had given clearance for five food items exposed to low doses of radiation for human consumption.

This method, which is patented, has been successfully used for cut roses, jasmine flowers, mogra and sayli. A method of preserving the fragrance of Indian varieties of jasmine is reported for the first time.

### Medium dose applications

Doses in the range of 1 to10 kGy were found to be useful to control pathogenic bacteria. The consumption of fresh vegetables, sprouts, fruits, meat and fish have been responsible for outbreaks of enteric infections in recent past. Several types of pathogenic bacteria such as *Salmonella, E. coli O157:H7, Shigella, Aeromonas hydrophila* and *Listeria monocytogenes* were responsible for these outbreaks. Use of irradiation to ensure hygienic quality of fresh, pre-cut fruits, vegetables, frozen sea foods, poultry and meat was tried. A dose of 2 kGy was sufficient to eliminate *Salmonella typhimurium* and *Listeria monocytogenes* and other pathogens without affecting organoleptic, nutritional and textural properties of minimally processed plant produce. Higher doses of 2 to 5 kGy were required for frozen sea foods, meat and poultry whereas doses of between 2 to 7 kGy were equired for improving juice yield and cooking quality.

### A radioresistant microbe *Deinococcus radiophilus* (ATCC 27602) was isolated in BARC in 1973

- Orange red pigmented colonies were isolated from Bombay-duck (*Harpodon nehereus*) fillets irradiated at 20 kGy dose of gamma radiation.
- The organism was named as *Micrococcus radiophilus* which was one of the most radiation resistant organisms both to UV and gamma radiation (Decimal reduction dose for gamma radiation-15 kGy).
- The organism was deposited in National Collection of Type Cultures, Colindale, London, as *Micrococcus radiophilus.*
- The organism was classified as *Deinococcus radiophilus* by Brooks and Murray in 1981.
- Later *D. murrayi* was shown to have higher radioresistnace.

### High dose applications

India is one of the largest exporters of spices. In early eighties studies on irradiation of spices were initiated. Spices were found to possess antifungal principles against aflatoxin producing fungi. These principles were not destroyed by gamma radiation. Moreover, Indian spices with high microbiological load could be easily and effectively hygienized by gamma radiation. A technology demonstration plant for this purpose was set up at Vashi, Navi Mumbai and is operational since January, 2000. High doses are also used for decontamination of vegetable seasonings, enzyme preparations and food packaging materials.

### Combining radiation preservation with other methods

A combination of high pressure treatment (3.5 kbar) and radiation (2 kGy) resulted in 4 -5 fold extension in the shelf life of shrimps as evaluated by freshness indices and absence of *E.coli*. Use of edible chitosan coating prior to radiation processing of intermediate moisture (IM) meat products eliminated the risk of contamination and spoilage of individual kebabs upon opening of the container. Extracts of potato peel, mango peel, mint leaf and chitosan have been found to be very potent natural antioxidants that could effectively minimize oxidative rancidity in radiation–processed meat and thus improve its keeping quality. Mechanisms underlying the effectiveness of these novel antioxidants were elucidated.

### Preservation of ready-to-eat products by irradiation

A number of shelf stable ready-to-eat meat products was developed using a combination of reduced water activity, packaging and gamma-radiation. The products had a good sensory acceptability and could be stored at ambient temperatures. The process was also found suitable for the preparation of high value seafoods such as room temperature stable shrimps that would be otherwise spoiled within a day.

### Preserving essential oils and phenolics after irradiation

Studies on the biogenesis and control of aflatoxin, a mycotoxin of economic importance in agricultural commodities, were also taken up. It was seen that naturally occurring sulphur compounds in onion, phenolics in potato and essential oils in spices displayed strong inhibitory activity against aflatoxin producing fungi. The activity of these natural defense compounds was not affected by radiation processing. Aflatoxin biogenesis was also seen to be inversely related to the concentration of fungal spore inoculum in a medium. Likewise, a reciprocal relationship between ethylene biogenesis and aflatoxin synthesis was discovered**.** These studies helped to establish microbiological safety of irradiated foods and strengthened the case for subsequent international approval of the technology.

### Food flavour and aroma chemistry

Analysis of food flavours and aroma of Alphonso mango and their quantitation were carried out to assess quality of fruits irradiated for delay in ripening. The aroma constituents of green mango were isolated and identified. Studies were extended to the biogenesis of flavour compounds *in vitro*. Similarly, work on characterization of aroma of Basmati rice was carried out. These studies were important in characterisation of aroma impact compounds in the commodities and would facilitate consumer acceptability studies.

Gamma radiation induced enhancement in the flavour of food products such as monsooned coffee, nutmeg, turmeric and pomegranate has been recently observed. The role of aroma glycosides in contributing to the enhanced aroma of these products was also established. In monsooned coffee, release of isoeugenol and 4-vinyl guaiacol from their glycosidic conjugates resulted in enhanced aroma of this product after irradiation (5 kGy) thus reducing the processing time for obtaining monsooned coffee of desired flavour and quality. In nutmeg, turmeric and pomegranate too the enhanced aroma upon radiation processing was demonstrated to be due to the release of characteristic aroma compounds from their bound glycosidic precursors. Hygenization of spice mixes such as sambar powder and coriander-cumin mix by gamma radiation (10 kGy) did not affect either their sensory quality or flavour with respect to colouring constituents as well as aroma principles.

### Wholesomeness and safety of irradiated food

In 1997 a joint FAO/WHO/IAEA study group on high dose irradiation evaluated wholesomeness of food irradiated with doses above 10 kGy. It concluded that food irradiated at any dose appropriate to achieve a technological objective was both safe and nutritionally adequate.To establish wholesomeness of irradiated food grains several generations of rats and mice were fed irradiated wheat for various periods. Possible mutagenic effects of irradiated food were also investigated. These studies, published in international journals of repute and later summarized in the form of a monograph, failed to show any adverse effects of irradiated diet.

*Radiation preservation of ready to eat foods*

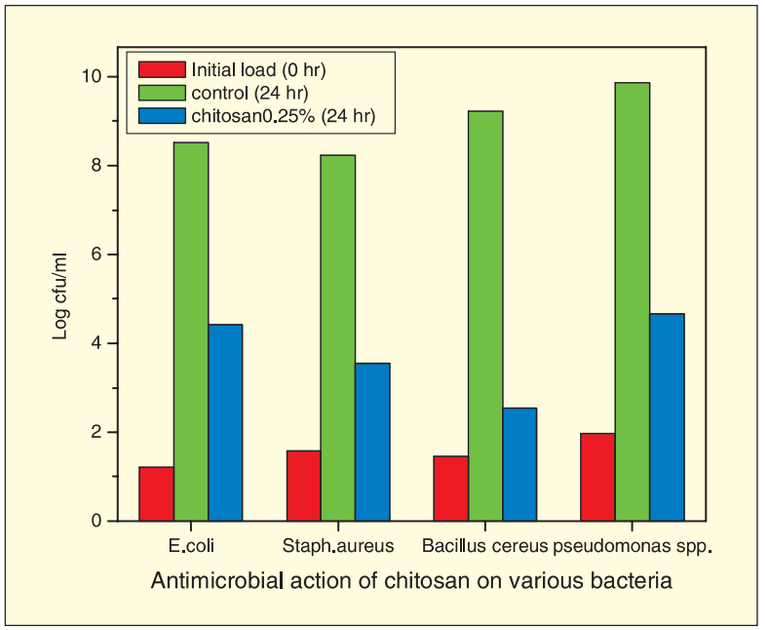
**General applications of food irradiation**

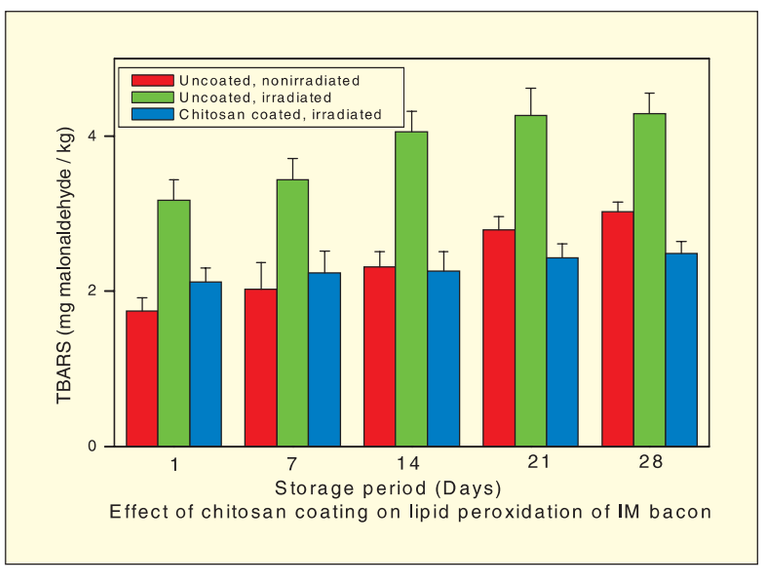
| Purpose | Dose(kGy) | Products |
|---|---|---|
| Low dose (upto 1 kGy) | | |
| Inhibition of sprouting | 0.05-0.15 | Potato, onion, garlic, ginger |
| Insect disinfestation and destruction of parasites | 0.15-0.50 | Cereals and pulses, fresh and dried fruits,dried fish and meat, fresh pork |
| Delay of physiological processes | 0.50-1.0 | Fresh fruits and vegetables |
| Medium dose (1-10 kGy) | | |
| Shelf-life extension | 1.5-3.0 | Fish, strawberries |
| Pathogens and spoilage organisms control | 2.0-5.0 | Frozen sea food. poultry and meat |
| Improve technological qualities | 2.0-7.0 | Grapes (increasing juice yield), dehydrated vegetables, pulses (reduced cooking time) |
| High dose (10-50 kGy) | | |
| Sterilization | 30-50 | Enzyme preparations, spices |
| Decontamination | 10-50 | Spices, enzyme preparations and natural gums |

**Food items approved for radiation preservation by the Ministry of Health and Family Welfare under Prevention of Food Adulteration Act Rules of 1955.**

| Name of food | Purpose | Dose (kGy) Mini-mum | Maxi-mum |
|---|---|---|---|
| Onion | Sprout inhibition | 0.03 | 0.09 |
| Potato | | 0.06 | 0.15 |
| Ginger | | 0.03 | 0.15 |
| Garlic | | 0.03 | 0.15 |
| Shallots (small onions) | | 0.03 | 0.15 |
| Mango | Disinfestation (Quarantine) | 0.25 | 0.75 |
| Rice | Disinfestation | 0.25 | 1.0 |
| Semolina (sooji, rawa) | | 0.25 | 1.0 |
| Wheat atta and Maida | | 0.25 | 0.75 |
| Raisins, figs and Dried dates | | 0.25 | 0.75 |
| Pulses, dried sea food | | 0.25 | 1.0 |
| Fresh sea foods | Shelf life extension | 1.0 | 3.0 |
| Frozen sea foods | Pathogen control | 4.0 | 6.0 |
| Meat and meat products and chicken | Shelf life extension & pathogen control | 0.25 | 4.0 |
| Spices | Microbial decontamination | 6.0 | 14.0 |

A process was standardized for bio-transformation of vanillic acid to vanillin using a fungus *Fusarium udum* isolated from a spice. Using this organisms a variety of other commercially important aroma compounds such as terpenes and lactones were obtained from cheap precursors.




*Development of edible active packaging using chitosan*

## Developing suitable packing material

Recent studies on the development of suitable packaging material for irradiated dried fish have indicated that the laminated structures (PET/POLY and PET/PET/POLY) provide a fairly acceptable shelf life for dried fish. Protein gel dispersion coating was found to be useful for extending the shelf-life of fresh water fish, rohu, in whole as well as steak form, and suppressed the microbial growth. Incorporation of antimicrobial peptide, nisin, eliminated *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli* from seer fish steaks during storage.

## Basic research in Food Science

Basic research in food science involved elucidatation of mechanisms of inhibition of sprouting in onion and potato, delay in ripening of tropical fruits such as mango and banana, inactivation of insects and microbes. Several new enzymes involved in ripening were isolated and characterized. In potatoes radiation was shown to induce transient metabolic activation, causing alterations in the synthesis of hormones and nuclear proteins. Cinnamic acid 4-hydroxylase, was purified from potato tubers and its non-microsomal nature was established. Delay in ripening of gamma-irradiated banana was attributed to reduced sensitivity to ethylene, the ripening hormone. Alterations in carbohydrate metabolism involving decreased ATP synthesis were also observed. Studies on solanin and chlorophyll synthesis in cold stored potatoes exposed to dim light revealed existence of a novel $C_1$ pathway for fixation of $CO_2$ in green potato tuber chloroplasts.

*Protein hydrolysate (right) prepared from Chicken intestine waste (left)*

Mechanisms underlying biochemical spoilage of fish and the role of hydrolytic enzymes were also investigated. Several lysosomal hydrolases were isolated, identified and characterized. By studying autolysis under different physiological conditions, involvement of lysosomal enzymes, especially proteinases, in causing spoilage of stored fish and meat products was established and a method for limiting their activities devised. An efficient autolytic method for retrieval of proteins and enzymes from meat, poultry and fish wastes was developed. Protein hydrolysates thus prepared were found to be nutritionally rich with good functional properties. Aspartic proteases, cathepsin L and aminopeptidases were isolated from meat and fish wastes and were characterised. Endopeptidases could be used for the preparation of

hydrolysates from soy proteins. Aminopeptidase was used for debittering the hydrolysates. Three species of *Acetes* (jawala), viz. *Acetes indicus*, *Acetes sibogae* and *Acetes johni* showed presence of cathepsin D, B, H and L, aminopeptidase, alkaline proteinase and azocoll lytic activities. Three enzymes belonging to serine proteinase family were purified to homogeneity from *A. indicus*.

### Enzymes involved in mango ripening

Cavendish banana and Langra mango, the important fruit crops remain green upon ripening at temperatures above 25-30 °C due to incomplete degradation of chlorophylls. However, on ripening at lower temperature the fruits developed full yellow colour. The enzymes involved were studied at different stages of ripening. In Cavendish banana, two different degradative pathways were operative for breakdown of chlorophyll a (Chl a). In Langra mango, pheophytin a and Chl b derivative were accumulated during ripening. These two compounds are slowly metabolized indicating that the stay-green character of Langra mango could be due to the accumulation of pheophytin a and the derivative of Chl b. Alphonso mangoes are prone to development of spongy tissue during ripening. Studies indicated that gamma irradiation at 0.1 to 0.3 kGy reduced the development of spongy tissue. Upon development of spongy tissue, the adjacent pulp turned black indicating involvement of phenolic compounds. The total phenolics, sugar concentration and soluble protein decreases in the affected fruits. Gamma radiation did not have any adverse effect either on sugar or phenolic constituents. In unripe fruits the peroxidase level was almost negligible. Ripening caused several fold increase in peroxidase activity. Spongy tissue affected fruits exhibited almost two fold increase in peroxidase activity as compared to ripe fruits. In irradiated fruits the enzyme activity was similar as that in unirradiated ripe fruits and expression of different isozymes of peroxidase was observed.

### Nutritional quality evaluation

Extensive studies were carried out to assess the nutritional quality of irradiated wheat. Radiation processing of soybean resulted in enhancement in antioxidant contents of this oil seed due to increased availability of free isoflavones following radiolytic breakdown of their glycosidic conjugates. Cooking facilitated extraction of phenolics. Qualitative TLC/HPLC analysis indicated that gallic acid was the major phenolic in the legumes besided catechin, caffeic acid, ferulic and vanillic acid. Gallic acid is an efficient free radical scavenger. Radiation treatment does not show any significant change in the composition and distribution in phenolics in all the legumes.

### Detection of irradiated food

Methods are being developed to detect the irradiated food to meet legislative requirements. A new reverse-phase TLC method was developed for separation and rapid detection of radiation specific hydrocarbons. A new fluorescent napthoquinone derivative identified in irradiated nutmeg was used to detect the treated spice. Electrical conductivity for potato and EPR for dry raisins, dates and dry fish and vegetables are the other methods that are under evaluation.

*Electronic Beam Irradiator*

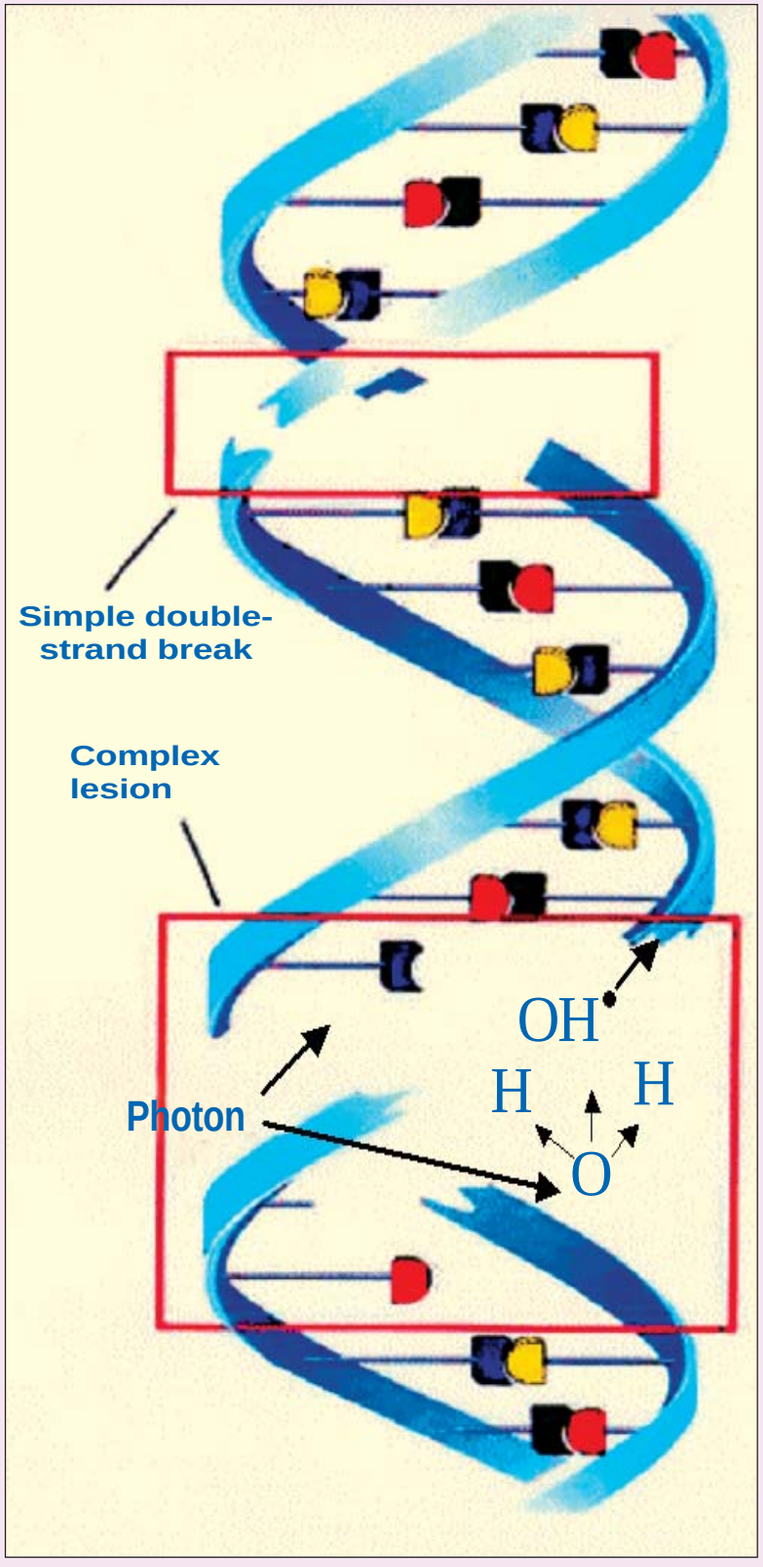
*Enzymatic extraction of fruit juices for health drinks and preservative free storage at refrigeration temperatures*

# Radiation Biology

## Link B4

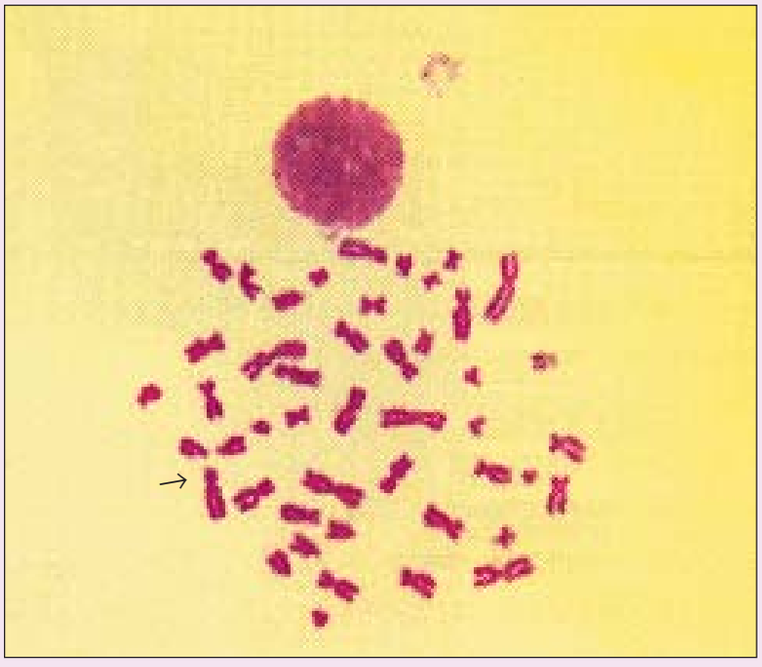
*Radiation doses of different magnitude, delivered at different rates to different parts of the human body, can produce different types of health effects at different times. There are radiations all over the universe. They have been with us since the beginning of time. These are classified as ionizing and non-ionizing radiations. Ionizing radiations include cosmic rays, X-rays and radiations such as alpha, beta and gamma emitted by radioactive elements. Non-ionizing radiations include UV, visible, infrared radiation, radiowaves and microwaves. Effects of radiation on living systems became a subject of urgent global concern after explosion of the atomic bombs at Hiroshima and Nagasaki in Japan at the end of the Second World War in 1945. The beneficial application of radiation in treatment of cancer and diagnostic as well as investigative medicine also became apparent around this time. It led to an exponential growth in biomedical research on applications of radiation and radioisotopes. A specially important contribution of radioisotopes is as tracers in biology, medicine and agriculture where they have been utilized to understand the metabolic fate of biomolecules, transport of nutrient elements in plants, mapping of metabolic pathways, nature of active sites of enzymes, cell proliferation kinetics, establishment of precursor-product relationship among proteins and molecular imaging of cancers. Interaction of ionizing radiation with living cells is central to modern radiation biology. In recent years new information, different models and ideas have accumulated concerning biological effects of ionizing radiation. Severity of radiation-induced damage usually depends on the absorbed dose and above a certain threshold (500-1000 mSv) the effects are detectable by laboratory or clinical methods. Such effects are called deterministic effects and involve cell killing. On the other hand, stochastic effects like induction of cancers and hereditary effects may occur at relatively low doses of radiation (100-200 mSv). In biological systems the indirect effects of radiation, mediated by free radicals produced due to radiolysis of water, predominate. Different types of dose response patterns have been reported for different end points. The probability of consequence increases with dose. However, for the purposes of radiation protection, the relationship between dose and effect is assumed to be linear. For certain effects a threshold has been observed and there are also several published reports in the literature, which indicate a possible beneficial effect at very low doses of radiation (hormesis). The uncertainties associated with effects of radiation at low dose rates and low total dose have posed several experimental as well as conceptual challenges.*

# Radiation induced DNA and chromosomal damage




*Breaks induced in DNA by direct and indirect action of radiation*

*Micronuclei produced due to chromosome breaks in cells after DNA damage*



*Formation of dicentrics shown by arrow*

# Radiation Biology and Health Sciences

Basic research in radiation biology was initiated in DAE under the dynamic and visionary leadership of Dr. A. R. Gopal-Ayengar. Some of the early studies showed that at least part of the radiation induced damage in biological systems can be attributed to intermediate molecular species. Elegant biophysical studies employing the technique of Electron Spin Resonance (ESR) revealed the role played by radiation induced free radicals. During the first 25 years, extensive biochemical investigations, with or without radiation stress, were carried out at BARC. These related to intermediary metabolism, vitamins (folic acid and vitamin B12), mitochondrial functions (bioenergetics), cellular development and differentiation (cancer biochemistry) etc. All these investigations (mostly mammalian biochemistry) employed the then prevalent sophisticated techniques of biochemistry and molecular biology. Later on, free radical induced damage to various cell constituents became the main focus. It was also realized that it may be possible to modify the extent or nature of radiation damage by pressing into service other molecular entities. Naturally, this stimulated search for such molecules. Several candidate molecules which could either enhance (radio-sensitizers) or reduce radiation damage (radio-protectors) were investigated in different systems ranging from cells to organs to whole animals. In this context, the possibility of using antioxidants for neutralizing radiation induced reactive oxygen species was also evaluated.

### Radiation targets in cells

DNA is considered to be the prime target for radiation damage as it is a crucial molecule for cellular function and survival. Nature has also provided mechanisms for repair of such damage. Radiation damage to DNA results in the formation of modified bases, single strand breaks (SSB) and double stand breaks (DSB). Radiation mediated DNA damage is believed to be linked to delayed DNA synthesis and inhibition of cell division. Radiation also affects cell membrane and organelles. Alterations in signaling, gene expression, cellular redox reactions and cell cycle regulation are observed. Membrane damage may become significant at non-lethal or moderate and therapeutic doses of radiation. Reactive oxygen species (ROS) are produced following exposure of cells to radiation, These have been implicated in damage to vital biological molecules producing changes such as genetic effects, cell killing and carcinogenesis. Radiation may also induce cell death either by a process of necrosis or apoptosis. The latter is due to programmed expression of certain genes. Radiation induced cell death can occur in resting cells (interphase death) or those undergoing cell division (mitotic death). Irradiated cells which escape death may undergo somatic mutation due to DNA damage and may be transformed into cancer cells.

### Biochemical studies with whole-body irradiated animals

Rats and mice exposed to doses ranging from 1 to 6 Gy died after about a month. However, within the first 24 hours there was enhanced synthesis of RNA and proteins in the liver but not in other organs like spleen or thymus. These biochemical changes were carefully followed using appropriately labeled ($^{14}C$, $^{32}P$ or $^{3}H$) compounds as precursors to trace their metabolic fate. These investigations helped the Labeled Compounds Section of the Isotope Division of BARC in developing their programmes for indigenous production of these radiochemicals for use by biology researchers in several universities and research institutions in India.

The enhanced rate of protein synthesis was found to be due to release of glucocorticosteroids. On the other hand, inhibition of RNA and protein synthesis was a reflection of reduced template activities of chromatin. These effects seemed to be at least partially due to indirect abscopal mechanisms. Protection of portions of the body containing these organs could not totally suppress the effects of whole body radiation exposures. Likewise, the radiation–induced suppression of DNA synthesis in the spleen and thymus could not be obliterated by shielding of the region containing these organs.

The accelerated DNA synthesis in the regenerating liver in rats after partial hepatectomy (surgical removal of a portion of the liver) was inhibited by whole-body irradiation at 10 Gy. These changes corresponded very well with the activities of DNA polymerase-$\alpha$ in liver nuclei and nuclear matrices (containing DNA replication enzymes and chromatin). Further investigations

**How do the living organisms maintain the integrity of their genomes?**

- It is now well known that organisms differ from one another in their DNA sequence. Genomes maintain the divergence as well as sequence integrity at the same time, a tightrope walk of regulation.
- Highly reactive environment, genotoxic agents, ionizing and non-ionizing radiation constantly challenge the genomic integrity by modifying the chemistry of genomes.
- Such onslaughts can result in altering the sequence and hence the final characteristic of the individual. Unrepaired DNA damage can also lead to genomic instability resulting in Cancer.
- It is estimated that thousands of nucleotides in the genome of a human cell are damaged spontaneously each day under normal physiological conditions.
- In case of exposure to ionizing radiations like $\alpha$, $\beta$, $\gamma$ rays, single strand breaks (SSBs), double strand breaks (DSBs) and base modifications are introduced in DNA. Exposure to UV radiation causes the formation of thymine-thymine (T-T) and thymine-cytosine (T-C) dimers.
- All normal living organisms are equipped with the capacity to repair this DNA damage, as "DNA is the only biomolecule that is specifically repaired. All others are replaced."
- Over 100 different kinds of proteins are recruited to take care of the health of DNA. These repair processes are conserved from simple bacteria like *E. coli* to very complex mammalian cells.
- It is, therefore, reasonable to conjecture that DNA molecules succeeded in becoming the genetic material of cells following co-evolution of robust repair machineries that constantly kept on correcting the instabilities.

revealed that whole-body irradiation blocked formation of DNA polymerase - $\alpha$ induction of which preceded the first wave of DNA synthesis at the onset of liver regeneration, thereby completely preventing the formation of DNA polymerizing apparatus. This effect on the DNA synthesis also seemed to be due to indirect physiological mechanisms.

DNA damage and repair were also studied in microbial systems, especially to understand mechanisms of radio-sensitization of microbial cells. The modes of radio-sensitization were different during irradiation under oxic and anoxic conditions. The work was also extended to mammalian cells in culture. A method for estimation of DNA strand-breaks using ultracentrifugation was developed. These efforts were useful in identifying certain unknown features of DNA-dependent functions. Repair of radiation-induced strand breaks in mitochondria was reported for the first time.

The phenomenon of respiratory adaptation, during transition of growth from anaerobic to aerobic environment, is associated with biogenesis of functional mitochondria in yeast. Anaerobic cells have a few rudimentary promitochondrial structures containing mitochondrial DNA. For biogenesis of fully functional mitochondria, replication of resident DNA is essential. This respiratory adaptation was found to be extremely sensitive to radiation/chemical mutagens. Elaborate studies on this unique finding showed that the system of respiratory adaptation could be used to test the ability of certain chemicals or physical agents to cause mutations. The test was found to be as effective as other established tests.

### Free radicals in radiation biology

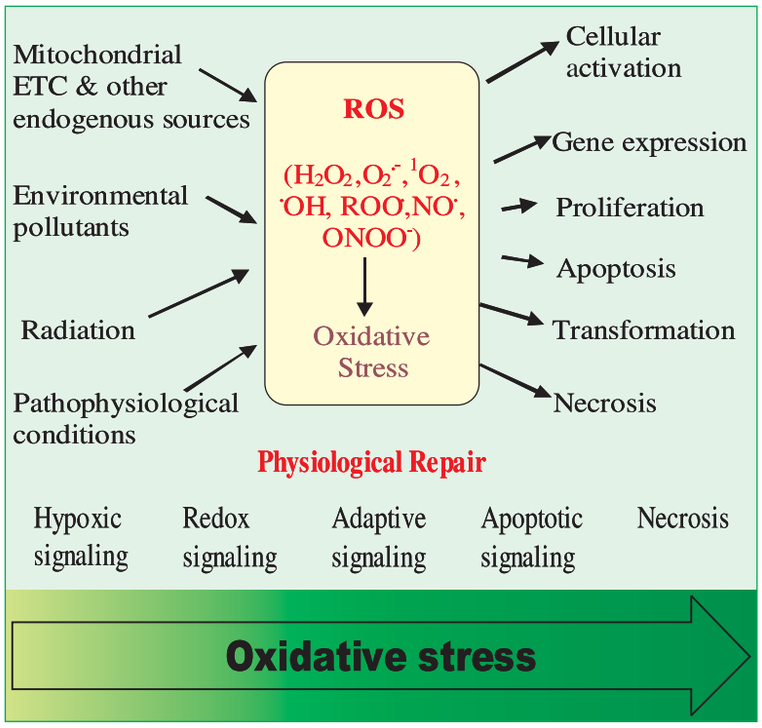
Radiation-induced free radicals bring about discernible alterations in cellular membranes. These and related biochemical reactions were seen to be playing crucial roles in normal physiological states such as postnatal development, ageing and pregnancy. Of particular interest were the reactive oxygen species (ROS) which include hydroxyl and peroxyl radicals, superoxide ions, $H_2O_2$ and singlet oxygen. They induced base damage and strand breaks in DNA and peroxidation of lipids and protein oxidation. Singlet oxygen was shown to be capable of inducing strand breaks apart from generating base alterations in DNA leading to mutations. A number of natural compounds were shown to be capable of protecting DNA against ROS

induced damage. These include curcumin from turmeric, the endogenous antioxidant lipoic acid and flavonoids that form important coloring pigments in the plant kingdom, caffeine from coffee, tea and cola-based soft-drinks, food flavoring agent vanillin, chlorophyllin, nicotinamide, carotenoids, polysaccharides from medicinal plants such as *Asparagus racemosus, Tinospora cordifolia* and extracts from *Terminalia arjuna* and other medicinal plants. Several model systems such as homogenates of liver, spleen, brain, mitochondria, pBR322 plasmid and lymphocytes have been employed in these studies. The assay methods include spectrophotometry, HPLC (DPPH assay), FRAP assay, assays of antioxidant enzymes like catalase, SOD and glutathione peroxidase. Pulse radiolysis and stop-flow techniques were used to arrive at reaction rate constants for the interaction of free radicals with the antioxidants. Cyclic voltametry gave the values of their redox potentials.

A given modifier may act as a radioprotector or a sensitizer depending on its concentration and cellular milieu. A case in point was provided by caffeine. In several systems, caffeine is known to potentiate DNA damage induced by a variety of physical and chemical agents. But it acted as a radioprotector in oxic conditions. Caffeine was shown to compete with oxygen for electrons, scavenged OH radicals and singlet oxygen. A very convincing evidence of radioprotective action of caffeine was obtained when at doses of 80 or 100 mg/kg body weight intraperitoneal administration of caffeine 60 minutes prior to whole body lethal exposure to gamma radiation resulted in the survival of 70 and 63% of the animals. The protection was 50% if the time interval between caffeine injection and irradiation was reduced to 30 minutes. Administration of caffeine three hours after irradiation did not protect the animals against lethality. Interestingly, intramuscular administration of the same dose of caffeine protected against radiation (35Gy) induced skin lesions. But intra-tumour administration in a mouse fibrosarcoma did not influence response of the tumour to local irradiation, indicating a potential application of caffeine in cancer radiotherapy.

The vitamin folic acid also displayed antioxidant and radio-protective properties *in vitro* which were attributed to its pseudophenolic moiety and C9 methylene group. The role of various enzymes in folate dependent one carbon metabolism related to DNA synthesis and repair was examined. Dihydrofolate reductase and thymidylate synthase activity increased up to 96 hr after whole body irradiation while that of methylene tetrahydrofolate reductase declined sharply. There was 54% reduction in total folates and p53 protein concentration increased. In lymphocytes chlorophyllin was shown to inhibit generation of radiation induced ROS and apoptosis.

The mechanism of radio protection against lethal doses of γ radiation offered by prior exposure to mild whole body hyperthermia (WBH) (39 $^{o}$C or 40$^{o}$C) was investigated. Nitric oxide was implicated in the modulation of thermal injury caused by WBH and elevated levels of immunomodulatory cytokines like IL-1α, IL-6, TNF-α and GM-CSF were shown to be responsible for protection and proliferative response of the bone marrow in mice treated with WBH and lethal irradiation. Radioprotective effects of disulfiram, troxerutin and tocopherol monoglucoside (water soluble vitamin E) against radiation damage to DNA and membranes were also demonstrated.




**Radiation damage to cell membranes**

Ionizing radiations are known to change permeability of cell membranes. Two major constituents of biological membranes viz. lipids and proteins are prone to oxidative damage. Events associated with membrane damage were shown to play a role in the development of radiation injury in cells including apoptosis in thymocytes and intestinal crypt cells.

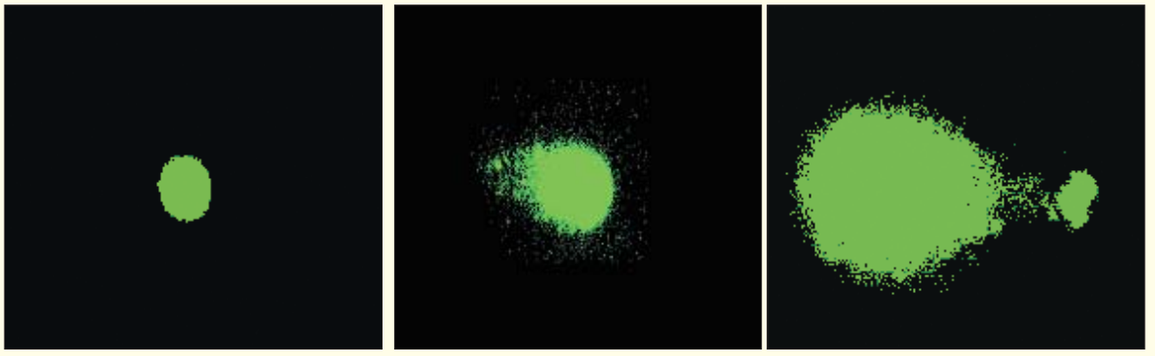
## Monitoring chromosomal damage

Extensive research was carried out to investigate effects of consumption of radiation processed foods on somatic and germinal cells of laboratory animals. Several assay systems like metaphase analysis and micronucleus assay for somatic chromosomal damage and dominant lethal mutations (DLM) for germinal effects were employed for the purpose. A number of radiation processed food products, such as wheat, onion, mackerel and paprika were evaluated for their genotoxicity in rats and mice.

Based on these studies the joint FAO/IAEA/WHO Expert Committee (1970), which reviewed data relating to the wholesomeness of irradiated wheat, concluded that extensive studies in animals fed wheat irradiated with gamma radiation dose up to 2 kGy had revealed no evidence that irradiated wheat was toxic or carcinogenic. These studies done in BARC contributed significantly and critically to the acceptance of safety of radiation processed foods by regulatory agencies, both at the national and international levels.

Scoring of chromosomal aberrations, deducing frequency of micronuclei as well as determination of DNA damage at single cell level by comet assay were used for evaluating genotoxicity of radiation and chemicals. PC based Digital Imaging Systems have gained wider attention for this purpose. Recently, three different software for cytogenetic and DNA damage analysis were developed. They are *Cell-Pro* for size measurement of cells, nuclei, micronuclei and cell/cytoplasm ratio, *Cyto-Pro* for numerical counting (e.g. aneuploidy and polyploidy) and karyotyping of metaphase chromosomes and *SCGE-Pro* to measure DNA damage using Single Cell Gel Electrophoresis (SCGE) or Comet assay. Significant increase in DNA damage at a dose as low as 1.25 cGy was observed.

### Comet assay for control and irradiated cells using *SCGE-Pro*



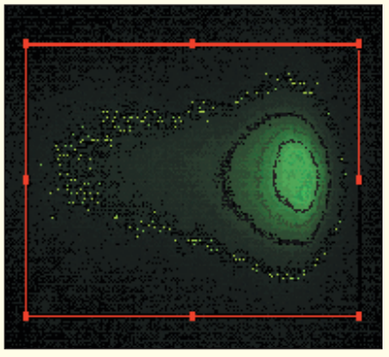
*DNA from undamaged peripheral blood leukocyte*



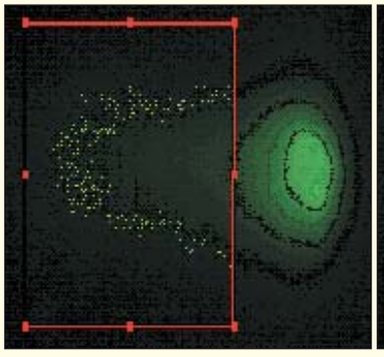
*Damaged DNA from peripheral blood leukocyte*



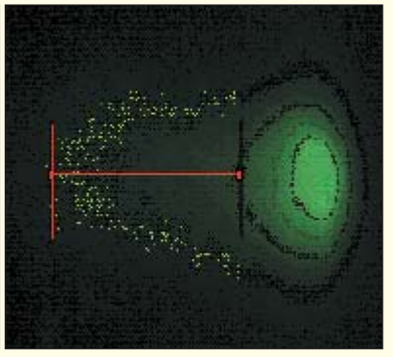
*Damaged DNA from an apoptotic leukocyte*



*Measurement of DNA fluorescence*



*Measurement of DNA fluorescence in tail*



*Measurement of migrated DNA fragments or tail length*

## Low dose radiation effects in animal models

Low dose radiation biology has attracted considerable attention in recent years. Low dose irradiation has no immediately noticeable effects on humans. There are very few hard observed data on cancer mortalty or other end points in the low dose exposure region (<100 mGy). There is considerable debate on the shape of the dose response curve in this region. Are these effects a simple extrapolation of those observed at high doses with severity varying as a function of dose or are there any safe limits below which the cellular defense machinery can carry out error free repair?

A wide spectrum of responses such as radiation hormesis (low dose exposure is beneficial), adaptive response (prior exposure to low dose protects against subsequent high dose exposure), genomic instability and bystander effects have been reported following exposure to low doses of ionizing radiation. There is a great interest in the "non targeted" effects of radiation (genomic instability and bystander effects) as these cannot be linked with the physical deposition of energy in the DNA molecule. These are not just challenges to the established paradigms of radiobiology but may also influence risk estimates following low dose radiation exposure.

## Radiation effects on immune system

Low dose radiation effects are being examined in experimental animals using cytogenetic, immunological as well as cell biological parameters. The immune system is critical for survival against infection. Incidentally, it is also the most sensitive to radiation. It is, therefore, not surprising that research on immunology and effect of radiation on immune response is being pursued in BARC. While there have been several reports demonstrating radiosensitivities of different cell types participating in immune response to different antigens, research efforts in BARC, led to evaluation of the radiosensitivities of such cell types in a single type of immune response (delayed type hypersensitivity) to a single specific antigen, a contact sensitizer, in a single strain of mice permitting comparative evaluation. Recent studies have addressed immunological consequences of low dose/low dose rate radiation exposure. These have, for the first time, demonstrated that fractionated low dose exposure to whole body gamma irradiation may augment or suppress immune response depending on the strain of the animals (mice), nature of immune response, the antigen and the total dose. While proliferation of lymphocytes in response to a polyclonal T cell mitogen was enhanced, expression of several critical proteins regulating cell cycle such as tumour suppressor p53, proliferating cell nuclear antigen (PCNA), retinoblastoma protein and D1/A cyclins was altered. Reduced expression of p53, less apoptosis and enhanced activation of $CD8^+$ T cells were also observed. Studies have also been initiated on the reconstitution of immune system in irradiated animals by homeostatic proliferation of transferred lymphocytes.

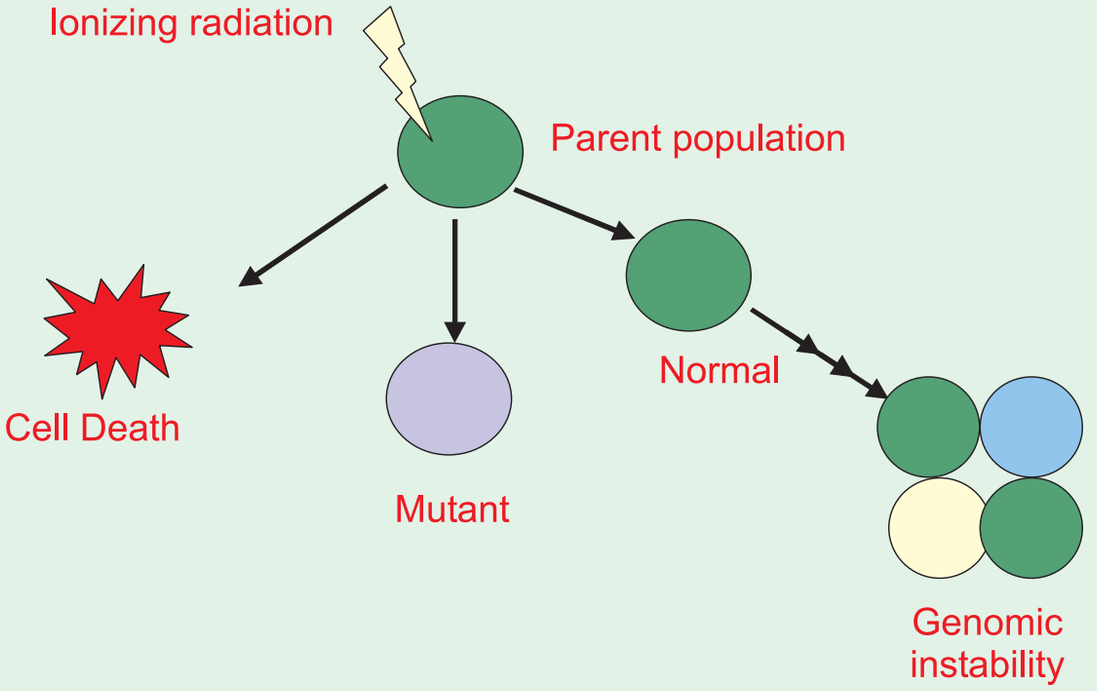
During recent years, it has been shown that adaptive response in prokaryotes may be mediated through the induction of DNA repair system(s). However, there is paucity of information, in human and mammalian systems, about the mechanism of adaptive response. Studies were carried out to understand the phenomenon of radio-adaptation in human lymphocytes of both sexes and different age groups using chromosomal aberrations and cytochalasin-B blocked micronucleus assays. Molecular mechanisms of radio-adaptation are being investigated. Adaptive effect was shown for the induction of a radiation induced tumour.

## Strategies for radionuclide decorporation

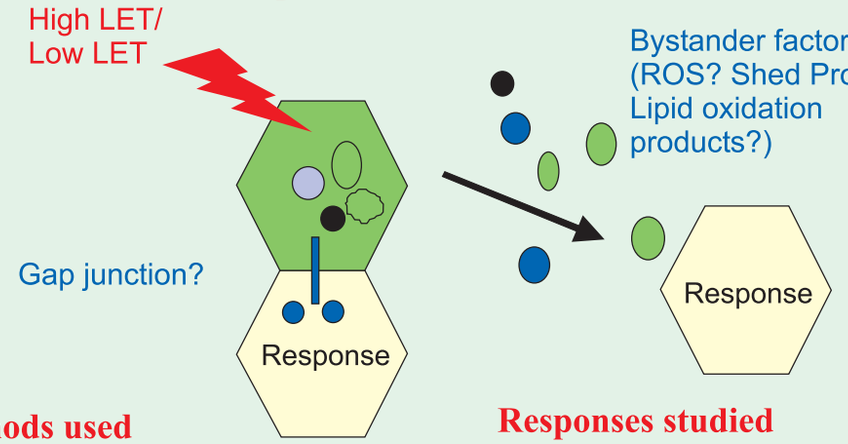
Removal and metabolism of bone seeking radionuclides strontium, radium, plutonium and thorium was studied in rats. These radionuclides are encountered in Nuclear Fuel Cycle. The physiological phenomenon of discrimination by animal body against very small amounts of strontium in favour of calcium was examined to develop a method for safe decontamination of strontium. Studies with radium showed that less than 0.2% of radium deposited in the maternal skeleton was transmitted to offsprings during pregnancy. Thorium could be removed by sialic acid apart from DTPA. A method was developed for rapid screening of potential decontaminating agents against thorium.

The Chernobyl nuclear accident has shown that only $^{137}Cs$ and $^{90}Sr$ deposited on the ground entered the body through the food chain apart from radioiodine induced damage to the thyroid gland in the early stages after the nuclear accident. Since both $^{137}Cs$ and $^{90}Sr$ can cause damage by the their propensity to distribute in tissues, simple compounds were indigenously developed which are more effective in reducing body burden of $^{137}Cs$ than the currently advocated decorporating agents.

# Genomic Instability




# Bystander Effects




**Methods used**

- **Micro beam irradiation**
- **Light weight ion irradiation**
- **Irradiated conditioned medium**
- **Coculture**

**Responses studied**

- **Gene induction**
- **Mutations**
- **Chromosome aberrations**
- **Apoptosis and cell killing**
- **Cell transformation**

# Cancer Biology

## Link B5

*Cancer causes only about 0.1% of deaths worldwide. Cardiovascular diseases result in higher mortality. Even so, the morbidity due to cancer and increasing cost of treatment make it the primary human enemy. Several agents such as chemicals, viruses and radiation are known to cause cancer. In addition, it is also a multifactorial disease. Besides the causative carcinogen there may be co-carcinogens and several promoting factors like food, lifestyle, smoking and genetic predisposition. Cancer is due to the failure of mechanisms that control proliferation and differentiation of cells. It arises from stem cells as also from other proliferating or non-proliferating cells. The process of carcinogenesis is a dynamic one where the genome is altered. The cancer causing genes (oncogenes) undergo mutation and transcribe proteins that are either over-produced or excessively active leading to continuous transmission of growth promoting signals. These, in turn, ensure viability and rapid division of cells. They also endow them with the capability to multiply in the absence of a growth factor, which is a prerequisite for normal cells. These rapidly dividing cells subsequently become resistant to signals that control cell death (apoptosis). Eventually this clone of cells forms a mass that invades surrounding tissues and establishes secondary areas of growth (metastasis). New blood vessels are formed to keep the tumour alive (angiogenesis). If untreated, it takes over the function of the organ or obstructs normal physiology finally resulting in death. Treatment modalities for cancer make use of its inherent characteristic of rapid cell division. These include chemotherapy and radiotherapy, the latter being the prime modality for treating primary tumors. However, it inevitably damages surrounding normal tissue that comes under purview of the delivered dose. Attempts are being made to minimize this damage by fractionation of the total dose to be delivered and by using various radioprotectors.*

# Mouse models for cancer

*Nude mouse models for gene therapy of oral cancer (squamous cell carcinoma of Pyriform Fosa and HSV/tk-GCV strategy)*

*ICRC mouse model for with esophageal cancers, Megaesophagous of ICRC mouse*

*Swiss bare mouse used to study skin cancers*

*Transgenic mice generated by injecting pK14-EF into single cell embryos to study squamous cell carcinoma*

# Cancer Biology

## Cancer research

Cancer research in India was first initiated at the Indian Cancer Research Centre (ICRC), established in 1952. ICRC was the brainchild of a great visionary in the field of Medicine, Dr. V. R. Khanolkar, who was the Director of Pathology and Biochemistry laboratories of the Tata Memorial Hospital (TMH). In the initial years, ICRC was under the purview of the Ministry of Health, Government of India. In 1966 it was rechristened as Cancer Research Institute (CRI). TMH and CRI were amalgamated to create the first comprehensive cancer centre in India, the Tata Memorial Centre (TMC). TMC itself became a grant-in-aid institution of the Department of Atomic Energy (DAE). ICRC was also the flagship of biomedical research in independent India. It, served as a cradle for several institutions - the Biomedical Group of Bhabha Atomic Research Centre, Institute of Immunohaematology and National Institute for Research in Reproductive Health. Cancer Research Institute is now a part of the Advanced Centre for Treatment, Research and Education in Cancer (ACTREC). Basic research in cancer was also undertaken in BARC and TIFR.

Earlier in 1961 epidemiological dental research was initiated in TIFR. Nearly 4700 policemen were examined to find out prevalence of oral leukoplakia, a precancerous lesion and its possible association with habits of tobacco chewing and smoking. A project for full scale epidemiological survey was then undertaken by Dr. Fali Mehta (TIFR) and Prof. J. J. Pindborg of Denmark. About 50,000 individuals over 15 years of age were examined in selected districts in the states of Gujarat, Kerala, Andhra Pradesh and Bihar. All important dental lesions were photographed with a Polaroid camera for instant documentation. The results showed that the habit of smoking with a clay pipe in Gujarat or of reverse smoking in Andhra Pradesh were associated with pre-cancerous lesions in the oral cavity. Prevalence of oral cancer was less at that time in Bihar as compared to Kerala.

Excessive, chronic occupational exposure to tobacco dust impairs those mechanisms in our body that inactivate harmful chemicals and thus elevate genetic damage, resulting in serious health problems. Ethnic differences in the susceptibility of an individual to oral cancer caused by tobacco were also documented. In parallel, alterations in cellular activity of the immune cells has provided leads to the susceptibility of individuals to cancer.

The dietary component turmeric (haldi) and its active principle curcumin were shown to have chemopreventive properties. Interesting leads are also expected for cancer chemoprevention with components of tea and grapes.

Early studies on oral cancer utilized experimental animals and focused on alterations in cellular components at the ultrastructural level. With the advent of the recombinant DNA era and the identification of genes involved in cancer, studies evolved from the cellular to the molecular level and the focus turned on to oncogenes and tumour suppressor genes. These studies provided a sequence of genetic events that lead from leukoplakia to frank oral cancer. Further, molecular cytogenetic studies have changed the focus from the chromosomal level to that of individual genetic regions/loci. Extensive immunological studies on the role of $\gamma\delta$ T cell receptor bearing cells in the oral cancer and the patients' peripheral blood lymphocytes have been carried out recently, which suggest a defect in their signal transducing ability.

## Gene therapy for head/neck cancers: 'Suicide' gene strategy

Chemotherapeutic regimens and radiotherapy have their limitations. To overcome these, gene therapy can be explored. A programme was, therefore, initiated to use a 'suicide gene' to kill oral cancer cells. Preclinical studies were based on prodrug activation strategy using the suicide gene - Herpes Simplex Virus–thymidine kinase (HSV-tk) and ganciclovir (GCV). A 2 kb sequence of HSV-tk was subcloned into the retroviral vector resulting in a recombinant vector, LTKSN, which was transfected into a packaging cell-line, PA317 and selected on G418. The highest expressing clone, PTK-16 was used for *in vitro* experiments. A xenograft model of human oral cancer was developed in nude mice for studies on gene therapy. Intra-tumoural injections of PTK-16 followed by GCV treatment showed significant reduction in the viable tumour volume ($p=0.009$). These results will form the basis for future clinical trials in HNSCC (head and neck squamous cell carcinoma).

### Clinical research on cancer

The Cancer Research Institute has been at the forefront of cancer research in India. Efforts were concentrated on some of the most common Indian cancer types like oral cancer which represents about 30% of all cancers occurring in males in India. It is associated with lifestyle factors such as the growing popularity of chewing tobacco, pan masala and gutkha. Well-designed animal studies showed that gutkha had tumor promoting as well as progressor activity suggesting that gutkha consumption might pose a carcinogenic risk among chronic habitués.

## Leukemias and lymphomas

Leukemias and lymphomas comprise about 10% of the total cancer burden and are subject of several investigations. Initially, the focus was on the role of blood groups and cancer.

Pioneering work related to population genetics in India was carried out in the early years. This led to identification of the Bombay blood group and the substance H in it.

Chronic myeloid leukemia (CML) is a haematopoeitic stem cell disorder characterized by occurrence of Philadelphia chromosome. This genetic anomaly results in various functional defects in mature myeloid cells, i.e. polymorphonuclear leukocytes (PMNL). These functions include cell adhesion, aggregation, chemotaxis, pinocytosis, phagocytosis, esterase activity, etc. PMNL of CML patients showed defects in polymerization of cytoskeletal proteins, actin and tubulin. All these functions were stimulated by a chemo-attractant - n-formyl peptide. Studies on the chemo-attractant receptor – ligand interactions showed lower expression and altered intracellular trafficking of classical chemo-attractant receptors – FPR and C5aR. Signal transduction events leading to actin polymerisation like activation of calcium ions, *ras* and *rho* GTPases were altered in PMNL of CML patients. Recent investigations have provided technology for the use of stem cells isolated from umbilical cord blood for the treatment of blood disorders.

Over the years, immunological aspects of leukemias and lymphomas, as well as functional alterations in leukemic cells have also received considerable attention. In the early 80's, monoclonal antibodies were being developed worldwide. A hybridoma laboratory was established which developed antibodies against myeloid leukemia cells and oral cancer antigens. Research is now directed towards using these antibodies, which have the potential for detecting epithelial cancers, for targeting drug delivery to cancer cells.

New mouse models have been established and characterized for their sensitivity to carcinogenic agents. One of these - the ICRC mouse with megaesophagus, was used to study esophageal cancers and the other - the Swiss bare mouse for skin cancers.

## Transgenic mouse

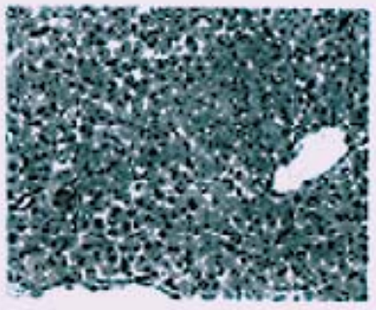
Transgenic mice were generated in ACTREC for the first time in the country for investigations of squamous cell carcinoma. An important molecule that controls growth/ differentiation of cells is called Enhancing Factor (EF), which modulates the action of Epidermal Growth Factor (EGF). It was isolated from mouse intestine. Transgenic mice expressing EF in squamous epithelial cells were generated by pronuclear injection of EF cDNA sub-cloned under a human keratin-14 promoter. These transgenic mice, TgK14EF, have abnormal hair growth and show expression of EF protein in skin (brown colour). Expression of EF coincided with hyperplasia. They were used to study the development of chemically induced squamous cell carcinomas using the two-stage carcinogenesis protocol. A statistically significant increase in tumour occurrence and reduction in time taken for tumour formation was noted in TgK14-EF mice, as compared to normal littermates. These mice also showed increased papilloma incidence and a 2-fold increase in the rate of conversion of papilloma to carcinoma upon application of a chemical carcinogen in the progression phase. These mice serve as good models to study chemical carcinogenesis.

With the advent of the Human Genome Project, the focus is now on providing global proteomic and genomic profiles of oral cancer. A few cancer related projects have also been pursued at BARC and SINP. These relate to understanding radiation resistance of tumours, use of liposomes and combination of electroporation and chemotherapy for cancer, understanding the differentiation between malignant and benign human breast tumours and measurment of the life span of leukemic leucocytes using radioactive phosphorous. In a recent study

## Effects of Gutkha extracts in ICRC mice

### Testicular tumour

Normal Tumour

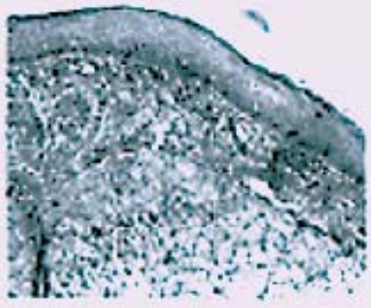


Leydig cell tumour

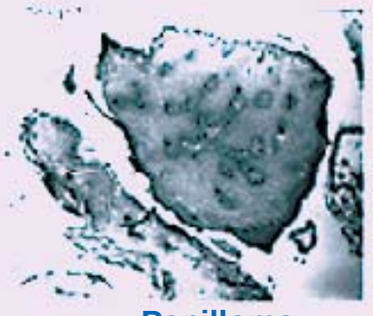
### Buccal mucosal lesions

Normal

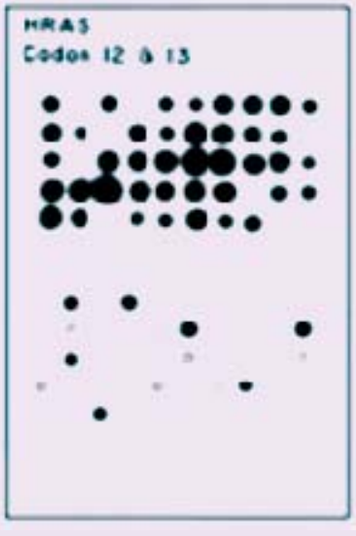
Hyperpiasia



Hyperpiasia + Dyspiasia

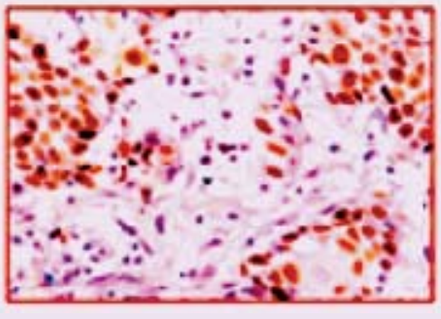


Papilloma




HRAS Codon 12, 13

erbB-1



Mutated p53

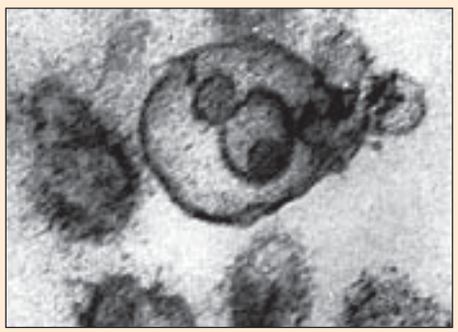
*Studying the expression of genes in oral cancer*

### Liver Cancer

High incidence of chronic infection with hepatitis B virus, which is linked to a 100-fold higher risk of developing liver cancer, necessitated in-depth molecular studies in this cancer, which indicate that the HBx protein plays an important role. Sensitive techniques such as RT-PCR and ELISA are being used to measure the presence of this gene and its product as potential markers to follow viral infection.

### Breast cancer

Breast cancer is a major cancer that affects females. It comprises 25% of the female cancer burden of the country. Genetic susceptibility and hormonal stimulation were found to play a role in breast cancer. Animal studies led to the development of the ICRC mouse model showing high incidence of breast cancer. One of the earliest classic contributions from the CRI was the identification of milk-borne tumor-inducing agents, later identified and characterized as mammary tumor viruses, leading to the recognition of the viral etiology of human breast cancer. About 5% of breast cancer patients show an early onset familial pattern. Studies showed that specific genetic alterations and weakened immune responses are potential markers for the identification of individuals at high risk for breast cancer.



*Particles isolated from milk of a breast cancer patient*

### Cervical cancer

Another major cancer affecting females is cervical cancer. Studies showed that analysis of human papilloma viruses (HPV), causally linked to this cancer, along with cytological screening of cervical lesions can help identify women with an apparently normal cervix who are at a higher risk of cervical cancer.

### Thyroid carcinoma

Thyroid carcinoma is a slow-growing tumour, which is well tolerated by the host. Probably immune mechanisms operate in human thyroid cancer to restrict tumour growth in some way. The immunological surveillance in thyroid cancer patients was studied using various parameters of cell-mediated immunity (CMI).

Amongst the different *in vitro* correlates of CMI studied, leucocyte migration inhibition test (LMIT), which detects the presence of sensitized lymphocytes, appeared to be most useful for detection and monitoring the course of disease provided a potent human thyroid tumour antigen was available. Several monoclonal antibodies to thyroglobulin were produced for use in immunoassays and immunohistology.

the higher sensitivity of normal lymphocytes to radiation induced apoptosis compared with that of leukemic cells was attributed to decreased generation of radiation induced ROS and enhanced antioxidant status in leukemic cells.

### Studies on cell cycle check points

Inheritance of DNA damage is a causative event in the progression of a normal cell to the transformed phenotype. The presence of damaged DNA in a normal mammalian cell leads to activation of checkpoint mechanisms that induce growth arrest and prevent cell division until the damaged DNA is repaired. Molecular mechanisms underlying checkpoint function in human cells, particularly regulation of the G2/M checkpoint by the 14-3-3 proteins are being investigated. Entry into mitosis requires activity of the Cdc25C phosphatase, which dephosphorylates and activates mitotic cyclin dependent kinase, Cdk1. Cdc25C, is a target of both DNA replication and DNA damage checkpoint pathways. Association of cdc25C with 14-3-3 proteins results in the retention of Cdc25C in the cytoplasm. It was observed that two 14-3-3 family members, 14-3-3e and 14-3-3g formed a specific complex with Cdc25C and inhibited its function *in vivo* while other 14-3-3 proteins were unable to do so even though they all formed a complex with cdc25C *in vitro.* These results suggested that other factors might

regulate specific complex formation between 14-3-3 proteins and their ligands *in vivo*.

## Signal transduction and radioresistance in cancer radiotherapy

The ability of a cell to respond to changes in its environment and to interact with its own counterparts, such that the whole tissue functions as a unit, is of paramount importance for viability of the organism. Signal transduction, as the name implies, is the passage of messages from the membrane to the nucleus of the cell and vice versa (intracellular) and also its communication with other cells (intercellular).

Transduction of the signal is possible due to highly specialized cell systems that integrate, transmit and amplify the available information. An understanding of the complex mechanisms responsible for these communications and their response under stress like carcinogen administration or radiation exposure will allow greater insight into the management of carcinogenesis as well as prevention of development of radioresistance following radiotherapy.

In recent years, the concepts of signal transduction and oncogenes have come to provide a common explanation for mechanisms by which agents as diverse as radiation and chemicals can induce tumours that are indistinguishable from one another. Ironically, treatment of these tumours with radiation often leads to resistance to subsequent radiation doses. Signal transduction is thus a very important area of research the world over.

As per our present knowledge, signaling cascades are primarily based on phosphorylation and dephosphorylation. The signal is passed to the nucleus via a number of modules, some carrying the message of cell survival and some for cell death. Studies with the carcinogens aflatoxin B1 and *N*- nitrosodiethyl amine indicated that aletrations of enzymes central to the second messanger system with resultant changes in phosphoinositide turnover would create conditions conducive to carcinogenesis.

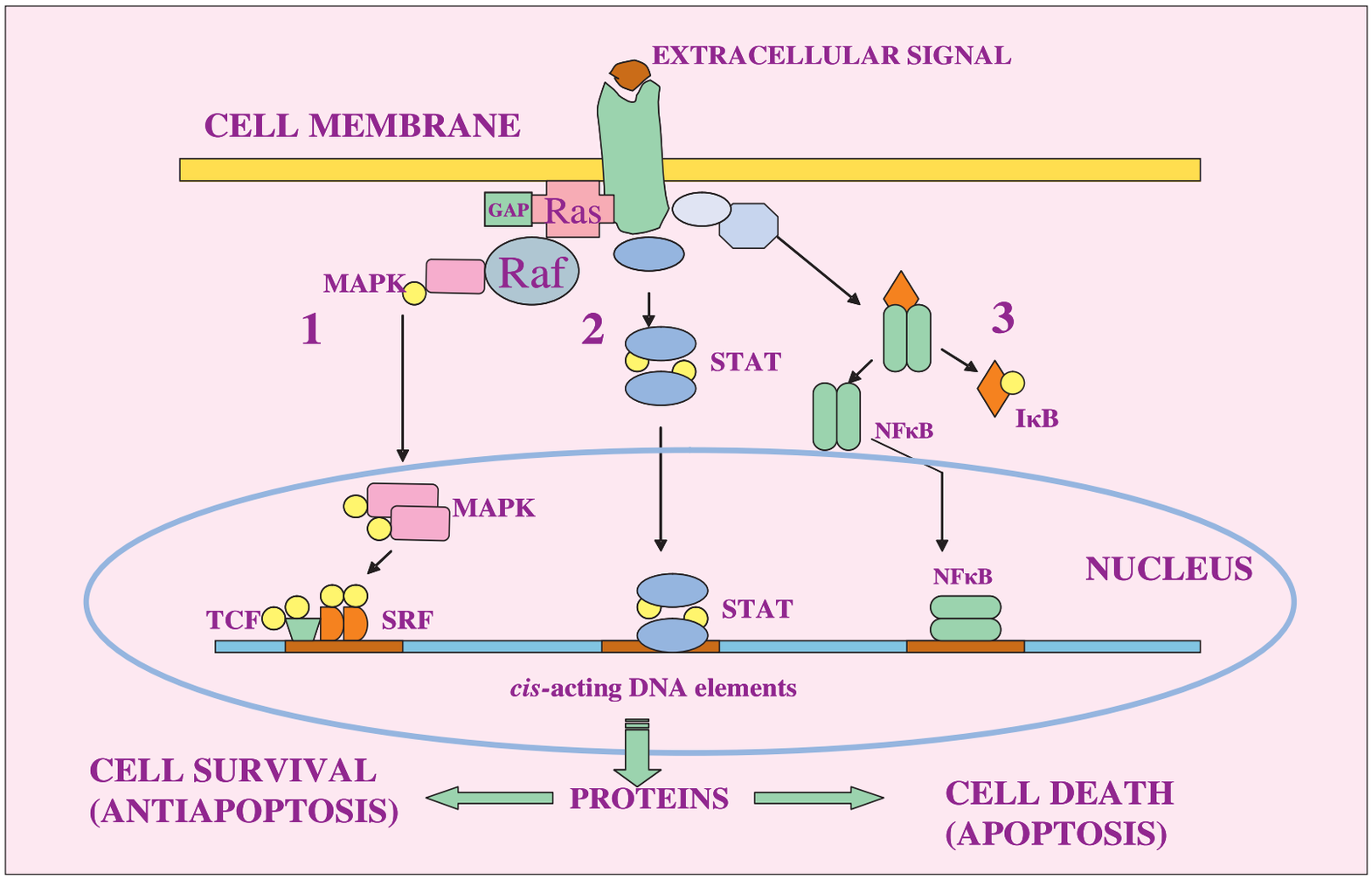
The *ras* signaling cascade where the gene undergoes a point mutation to become an oncogene is very important in carcinogenesis. Association of ras protein with a GTPase activating protein (GAP) enhances its GTPase activity several fold. Carcinogens (like N`N nitrosodiethyl amine) were found to reduce GAP activity significantly. Overexpression of the oncogene was, therefore, found to be accompanied by another aberration in the cell i.e. suppression of an inhibitor (GAP). This was brought about by extensive phosphorylation of GAP by protein kinase C (PKC), a very crucial enzyme in signaling that cross-connects a number of pathways. It also phosphorylates and activates crucial DNA synthetic enzymes following carcinogen exposure. PKC plays a role in mitogenesis (cell proliferation) as well as apoptosis (cell death).

A large body of evidence suggests that involvement of activation of signal transduction cascades upon irradiation or up-regulation of growth factor mediated pathways due to oncogene transformation often contributes to an acquired or inherent treatment resistant malignant phenotype. With this in view the focus was shifted to identifying signaling markers of radioresistance and their modulation or inhibition to prevent development of radioresistance following radiotherapy.

Radiation-induced signal transduction leads to simultaneous activation of certain nuclear alarm signals, which are a consequence of DNA damage. They also bring about activation of membrane bound and cytoplasmic signaling factors like PKC, MAPK, tyrosine kinases etc. One noteworthy finding was that expression of these factors was different following *in vivo* and *ex vivo* exposure to radiation. Some survival factors like MAP kinase (MAPK and ERK1/2) and nuclear factor-kB (NF-kB) were found to be expressed as late as 7 days after irradiation. The PKC isozymes, which can either function as oncogene or antioncogene, were differentially activated following irradiation.

Many pharmacological agents that inhibit specific key entities of these signaling cascades potentially sensitize them for radiation induced cell death. Hence, interference with the activity of these signaling molecules represents a novel form of experimental cancer treatment that may simultaneously restrain the proliferative state and invasive capacity of the high-grade tumours. Various synthetic compounds, which inhibit the kinases, are being tested for their efficacy in inhibiting cell survival components (PKC, MAP Kinase, NFkB, etc.) of the signaling system. Their poor selectivity and toxicity have shifted the attention to natural modulators as inhibitors of carcinogenesis. Many flavones, carotenoids and vitamins were found to be effective.

## Three pathways of information flow




The phenomenon of chemical radiosensitization is important for radiotherapy of cancer in order to bring about reduction in the effective dose. It was found that the quality of cellular damage resulting from irradiation under anoxic conditions does not change by inclusion of radiosensitizers during irradiation. However, if cells are irradiated in presence of oxygen the quality of the cellular damage is different in the presence of radiosensitizers. Misonidazole, a nitroimidazole drug used to treat protozoan infections was reported to be an effective radiosensitizer under hypoxic conditions. The great promise of Sanazole (N-2'-methoxy ethyl)-2-(3''-Nitro-1''triazolyl) acetamide, a nitrotriazole compound was confirmed.

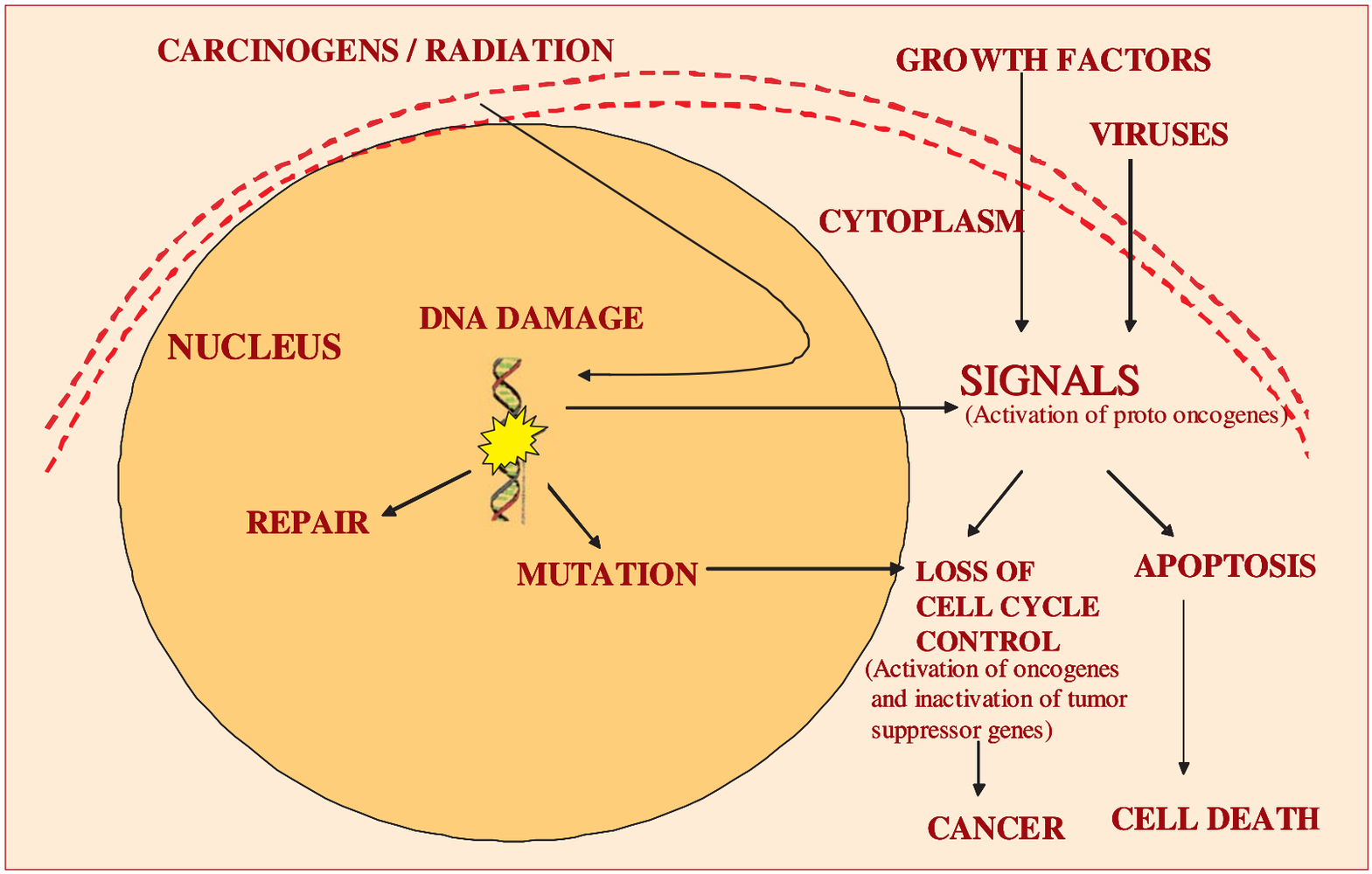
Radioprotective compounds are also of importance in clinical radiotherapy. In order to obtain better tumour control with higher doses, normal tissues should be protected against radiation injury. Search for more effective and less toxic radioprotectors has led to increasing interest in natural as well as synthetic pharmaceutical compounds with low toxicity profiles, which can suppress formation of free radicals.

### Lasers in cancer diagnosis

Laser Induced Fluorescence (LIF) in endogenous cellular constituents as well as using fluorescent drugs like haematoporphyrin derivative, which are selectively retained by the tumour, is being developed as a tool for diagnosing cancer. Studies carried out over the last few years showed considerable promise of this approach for diagnosis of the cancer of uterine cervix, esophagus, lung, breast, and oral cavity.

While malignant breast tissue sites (invasive ductal carcinoma) were considerably more fluorescent than benign tumour (fibroadenoma) and normal tissue sites (uninvolved region of the resected tissue), reverse was the case with the

## Stress induced signaling pathways




tissue from oral cavity. For the latter, malignant sites (squamous cell carcinoma) were considerably less fluorescent than normal tissue. Various measurements on tissue fluorescence (excitation-emission spectroscopy, synchronous scan and time resolved measurements) were carried out to unravel reasons for the observed difference in fluorescence from normal and malignant sites. These suggest significant variation in the concentration of fluorophores in different tissue types. In particular, these studies showed that while concentration of NADH (reduced nicotinamide adenine dinucleotide) was higher in malignant breast tissues compared to benign tumour and normal breast tissues the reverse was true for tissues from oral cavity where NADH concentration was higher in normal oral tissues.

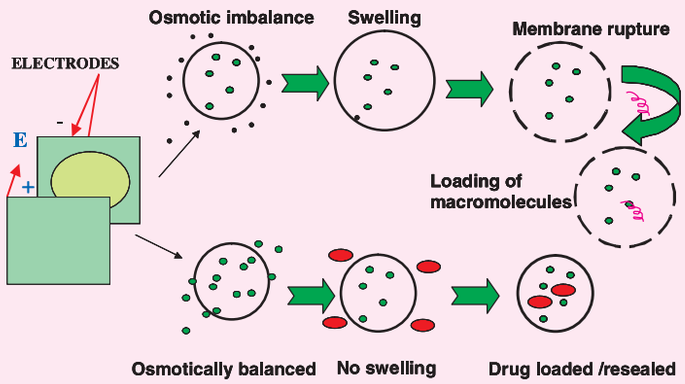
### Studies on photodynamic inactivation of cancer cell lines

Singlet oxygen is toxic to normal cells, but its selective generation can be the basis of cancer therapy (Photodynamic therapy). Investigations were also carried out on photodynamic effects of various photosensitizers (MC540, ALA, Cholrin p6, etc) on cell lines of epithelial neoplasm. Merocyanine 540 (MC540) was found to be effective for photoinactivation of carcinoma of cervix (HeLa) cells and plasma membrane was found to be the primary target for photodamage. The photodynamic effect of chlorin p6, a photosensitizer prepared in-house from chlorophyll-a, on two mammalian cell lines, human colon adenocarcinoma (Colo-205) and adenocarcinoma of breast (MCF-7), was also investigated. Significant differences were observed in the pH dependence of uptake and photosensitivity of chlorin p6 in the two cell lines.

Over the years, work on several projects, that are unrelated to cancer but are of medical relevance and national importance, have also been carried out at CRI. Bhopal gas tragedy victims were examined for mutagenic, genotoxic and teratogenic effects of methyl isothiocyanate. A national facility for anti-cancer drug screening using a panel of human malignant cell lines has been established, which also has an *in vivo* drug testing facility that uses murine tumours and human tumour xenografts in nude mice.

### Electroporation in tumour therapy

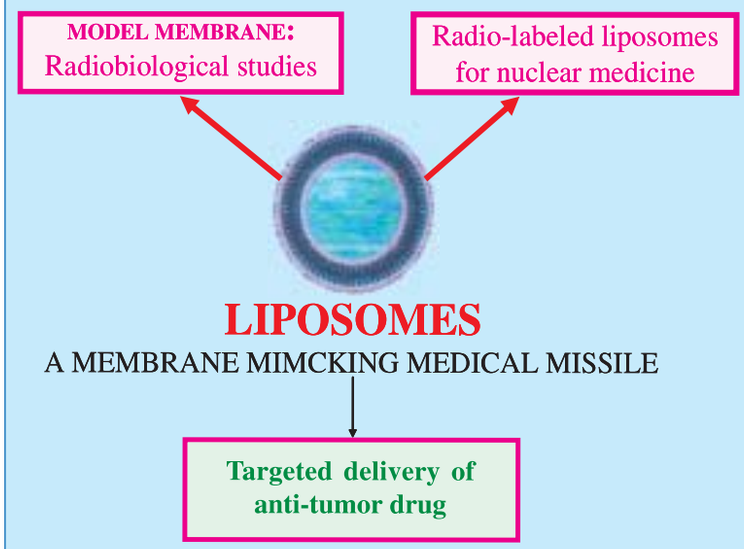
Radio-cytotoxicity towards tumour cells can be remarkably enhanced by electroporation. These studies are being carried out using a prototype Biphasic Medical Electroporator. It has potential in gene delivery, drug loading and cell manipulation. Electroporation methodology was employed to overcome resistance of uptake of radioiodine in a thyroid cancer cell line which allowed incorporation of substantial radio-iodine in otherwise impermeable cells. It was demonstrated that radiocytotoxicity to tumour *in vitro* could be increased by electroportaion.



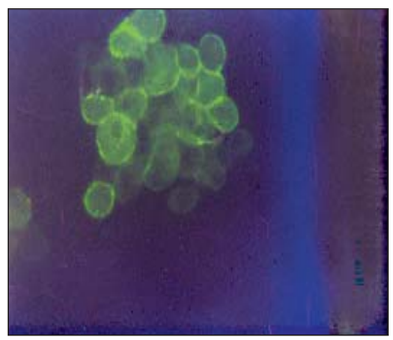

*Cell electroporation is the physical process of inducing transient permeability in plasma membrane by high intensity and short duration electrical pulses.*

### Drug delivery and drug targeting for cancer radiotherapy

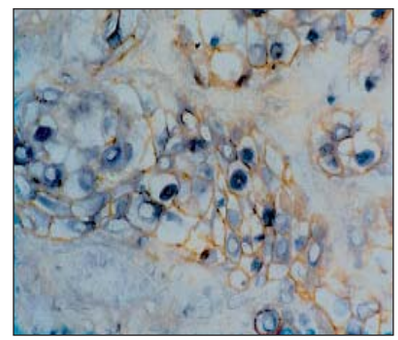
Phospholipid liposomes offer a unique bag with potential to package and transport anticancer drugs and radio-pharmaceuticals to disease sites. BARC has contributed significantly to develop, characterize and use liposomes for delivery of anticancer drugs. Stealth liposomes with PEG and having temperature sensitive response have been designed and developed for targeted delivery of drugs. It has been demonstrated that combination of TSL and hyperthermia in transplanted fibrosarcoma on mice produced more than 50% regression of tumor over each of the controls. Radiolabeled liposomes have been developed for diagnosis and therapy of cancer.




*Liposomes act as Bio-capsules, formed by the self assembly of the phospholipid molecules in an aqueous environment*



*Binding of* MAb3F8E3 *to oral cancer cells as analysed by immunofluoresence staining*



*Binding of* MAb3F8E3 *to oral cancer cells as analysed by Immunoperoxidase staining*

# Nuclear Medicine

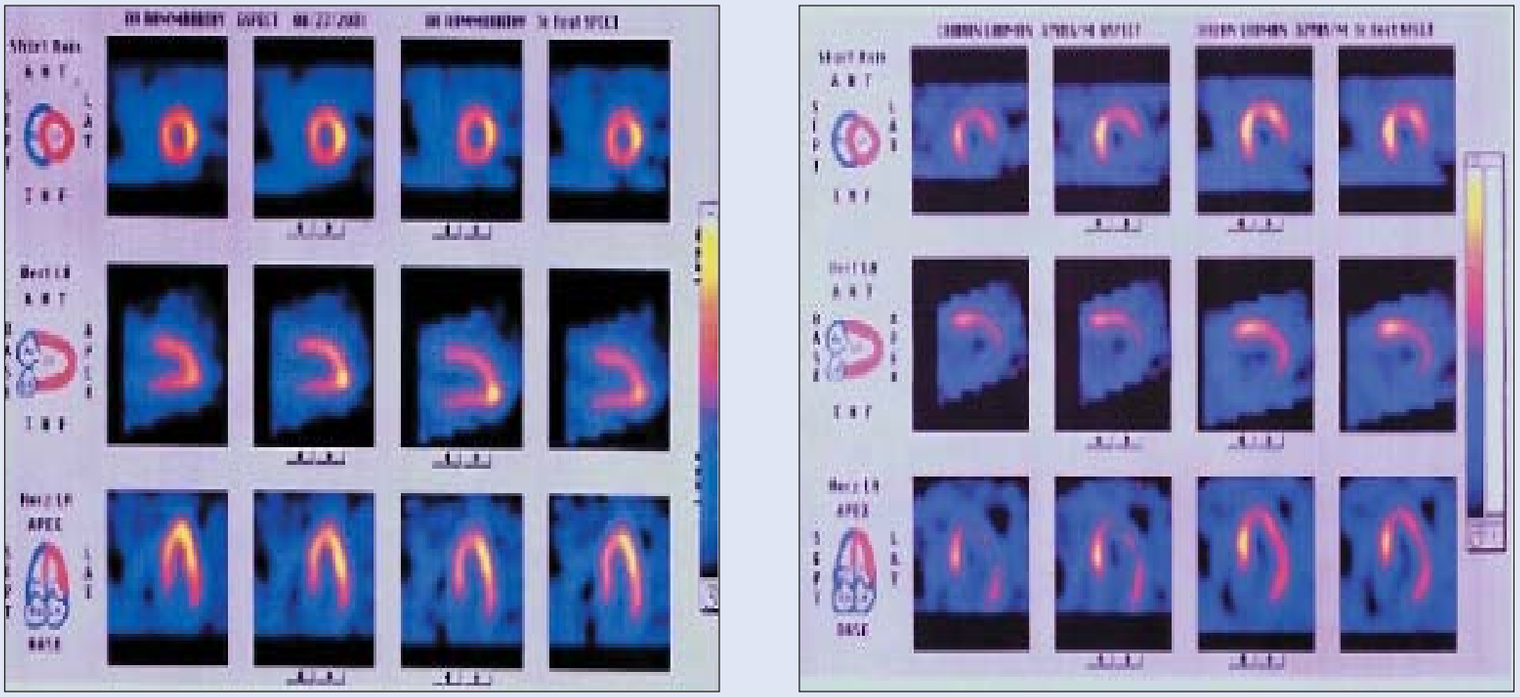
## Link B6

*Isotopes are atoms of the same element, which have identical number of protons in their nucleus but differ in the number of neutrons. Consequently, they have the same atomic number but different mass number. Isotopes of an element have the same electronic configuration and hence the same chemical properties. Isotopes of many naturally occurring elements are stable and do not undergo change with time. On the other hand, there are some naturally occurring isotopes, which are unstable and emit nuclear radiation. They are 'radioactive' and hence their presence in matter can be easily traced. With the availability of nuclear reactors as sources of neutrons, it became possible to artificially produce radioactive isotopes, by irradiating different elements in the reactors. These radioactive isotopes could be used as tracers in chemical and biological reactions. They constitute important tools for diagnosis as well as therapeutic treatment, particularly for cancers and tumours. A new specialty in medicine called 'Radiation Medicine' was thus born. The discovery of radioimmunoassy (RIA) in which a radiolabeled antigen competes with its unlabeled counterpart present in the biological fluids like serum for binding to a specific protein called antibody, marked a major turning point in diagnostic medicine. It made possible the estimation of several biomolecules like hormones, neurotransmitters and peptides which were present in small quantities in sera of patients. The RIA kits and radiopharmaceuticals revolutionized the medical world. Today, with the availability of cyclotron produced radiopharmaceuticals like $^{18}$F 2-fluorodeoxy glucose (FDG), functional imaging of tumours and other lesions is possible which has made a tremendous impact on therapy.*

# Radioimaging at Radiation Medicine Centre

*Radioimaging at RMC in early days*

*Positron Emission Tomography (PET) the most advanced diagnostic tool for cancer, cardiac, neurological and psychiatric disorders, avaliable at RMC*



*Perfusion images of heart using $^{99m}$Tc-MIBI. The series of pictures on the left are normal images and on the right show perfusion abnormalities*

# Nuclear Medicine

The proposal to set up Radiation Medicine Centre (RMC) in DAE was made by Dr. Bhabha in 1958. Commissioning of Apsara and CIRUS reactors provided the necessary impetus by facilitating indigenous supply of radioisotopes. This marked the beginning of research in Nuclear Medicine in India.

Several radiopharmaceuticals were first made by Isotope Division in BARC and supplied to different hospitals in the country. Necessary research, development and evaluation work is carried out in Radiopharmaceuticals Division, RMC and Laboratory Nuclear Medicine Section at BARC. Basic research on thyroid diseases and malabsorption of vitamins was initially undertaken in RMC. It also participated in the development of nuclear instruments in BARC, which were useful in detection of radioisotopes. Radioisotopes of indium and technetium were introduced in the next decade. In the last several years, radioactive technetium ($^{99m}$Tc), which is cheap and can be produced in large scale, has become a work-horse in radiation medicine. Several technetium labeled compounds are in use as radiotracers. Gamma camera was used and improvised for counting single photons. RIA kits are being produced since 1968. A lung ventilation system, a scanner for generating profile of whole body, a dual probe renography unit and impedance plethysmograph were also developed.

## Biomedical investigations on humans and animal models

The 1960s and 1970s saw a tremendous expansion in diversified fields of laboratory medicine. Development of newer techniques and refined procedures for isolation, purification and analysis of biomolecules, sophisticated techniques for immunodiagnosis and production of highly specific monoclonal antibodies along with sensitive tracer techniques have opened up new vistas in various disciplines of clinical medicine.

Before the advent of short-lived $^{99m}$Tc, classical radioisotopes were used. Initially the work was confined to tropical diseases, especially tropical sprue (TS), protein losing gastroenteropathies (PLG) and malnutrition syndromes. Using $^{14}$C-$HCO_3$, an original method to measure rates of *in vivo* synthesis of liver-formed plasma proteins was developed. To estimate gastrointestinal protein loss in patients with TS and PLG, a new radiotracer $^{95}$Nb-albumin was shown to be reliable as it was not absorbed and could be recovered in the stools. Simultaneously, for the first time, rates of synthesis and turnover of albumin and fibrinogen were measured in these patients and the effect of antibiotics studied using $^{14}$C-$HCO_3$ and $^{131}$I-labelled HSA.

### WHO centre for thyroid diseases

RMC is a WHO recognized centre for studies on thyroid diseases. RMC was among the first in the country to standardize and use the T3-uptake test. However, being an indirect measure of circulating thyroid hormones, this test was soon replaced by specific RIA for T3, T4 and TSH. RMC has come a long way from the primitive technologies used then to the present day solid-phase-antibody assays and semi-automation to make the assays user-friendly and faster.

To get a better understanding of the etiology and pathophysiology of various diseases, a number of animal models were developed for human disease conditions such as inflammation, alcohol induced liver diseases, xenobiotics-induced liver cirrhosis, chemical-induced hepatitis, filariasis, malaria, hypothyroidism, hyperthyroidism, thyroid tumours, goiters and chemical thyroidectomy. Further, these animal models were used to elucidate biokinetics of radiopharmaceuticals (RPs). The latter studies showed that the expected localization of a RP can be altered if there was a significant change in the biochemical milieu due to a disease. A case in point is the behaviour of $^{99m}$Tc-RPs viz., sulphur colloid, phytate, BULIDA and DIPIDA due to altered blood components in cirrhosis.

Liposomes of different sizes and electrical surface charges were prepared and used to encapsulate various isotopes and radiopharmaceuticals and their distribution in laboratory animals and animal tumor models was studied as a potential tool for cell-specific scintigraphy. Although propyl thiouracil (PTU) and methyl mercaptoimidazole (MMI)/neomercazole are in clinical use as antithyroid drugs for more than five decades, in the 1970s many facets of their biochemical actions on the thyroid and extrathyroidal tissues were not clear. *In vivo*, PTU was

found to be more potent than MMI. There were subtle differences in the mechanism of their action on thyroid and extra thyroidal tissues, principally, the liver. Unlike MMI, PTU showed significant and potent extra thyroidal action unrelated to its primary action on the thyroid gland.

### Calcium and vitamin D metabolism

It was observed that increased demand for $Ca^{2+}$ and inorganic phosphate (Pi) during physiological conditions of stress such as pregnancy, lactation and growth were met with the increased production of vitamin D hormone (Calcitiol). Hypophysis and vitamin D/PTH endocrine system have an independent role in the mobilization of bone minerals particularly under conditions of physiological stress. In this regard it was demonstrated for the first time that prolactin, which is maintained at the elevated levels during pregnancy and lactation, could mobilize $Ca^{2+}$ and Pi from bone and intestine without involvement of vitamin D/PTH endocrine system. These studies helped in elucidation of certain disease states of reduced bone mineral density in hyperprolactinemia in women due to amnorrhea leading to more effective treatment.

Another major finding was that vitamin D and calcium had an important role to play in the modulation of hepatic function and anti-oxidant defence system. Increased lipid peroxidation and reduced levels of hepatic anti-oxidant enzymes, particularly superoxide dismutase and glutathione peroxidase, were observed in experimental vitamin D deficiency. Histopathological changes leading to peri-portal necrosis were seen in these animals. These changes could be reversed to normal with the supplementation of vitamin D and calcium.

Although calcium salts are in clinical use from time immemorial the remarkable influence of anionic association of calcium on its bio-availability in the *in vivo* milieu was demonstrated for the first time. This has provided useful guidelines for extra calcium supplementation, particularly in better management of patients who develop hypocalcemia following thyroidectomy due to thyroid cancer. It was also demonstrated that increased intake of calcium helped in reducing incidence of stone formation by increasing excretion of the chelated complexes of oxalates and citrates (from the diet) through gastro-intestinal route.

### Laboratory evaluation of thyroid function

Free thyroid hormones (FTH) are physiologically active components. Still they constitute a very small part of the total thyroid hormones that are (~0.02% for FT4 and ~0.3% for FT3) in thermodynamic equilibrium with total T4 (9.98%) and T3(99.7%). Assay techniques for free hormones require a theoretically and conceptually different approach as compared to classical RIA. Using a two-step back-titration method with high affinity antibodies on a solid phase and a high specific activity tracer, assays for both FT3 and FT4 were standardized. Another method based on Sephadex column chromatography followed by RIA was also standardized wherein the BRIT RIA kits for total hormones could be used with modifications. Both the methods are comparable and were validated clinically. A mismatch between $T_4$ and TSH was shown to be due to thyroid hormone binding protein (THBP) abnormalities. A large study has shown prevalence of TBG deficiency to be somewhat higher in Indian patients compared to that reported in literature.

To understand systemic effects of non-thyroidal illnesses on thyroid function, tests were carried out in patients with renal failure and liver diseases and animal models with hepatitis. T3 suppression was seen and could be correlated with severity of illness while T4 suppression indicated morbidity. The role of thyroproteins in thyroid hormone synthesis was established. However, physiological role of thyrolipids was not clear in the early ’70s and ’80s. Studies in RMC indicated that apart from the lipid-iodide complex, lipid-iodine bond was also formed during iodine metabolism. The rate of iodination of lipid goes hand in hand with that of thyroid hormone formation indicating interrelation of the two phenomena. Mitogenic effect of T4, but not T3, was demonstrated on fibrosarcoma in Swiss mice. Tumorigenic effect at the nuclear receptor level could be the cause. The tumours grew faster in males than females.

### Mucociliary clearance studies

Innovative application of the radioactive-aerosols was developed for studying pulmonary ventilation and mucociliary clearance of particulate pollutants deposited in the lungs during respiration. A new aerosol delivery system was developed using larger sized particles of 2-5 mm AMAD (Activity Median Aerodynamic Diameter) of human serum albumin. Ciliary movement in terms of MTTR was measured by a computer

## Radiotracers used in radiation medicine

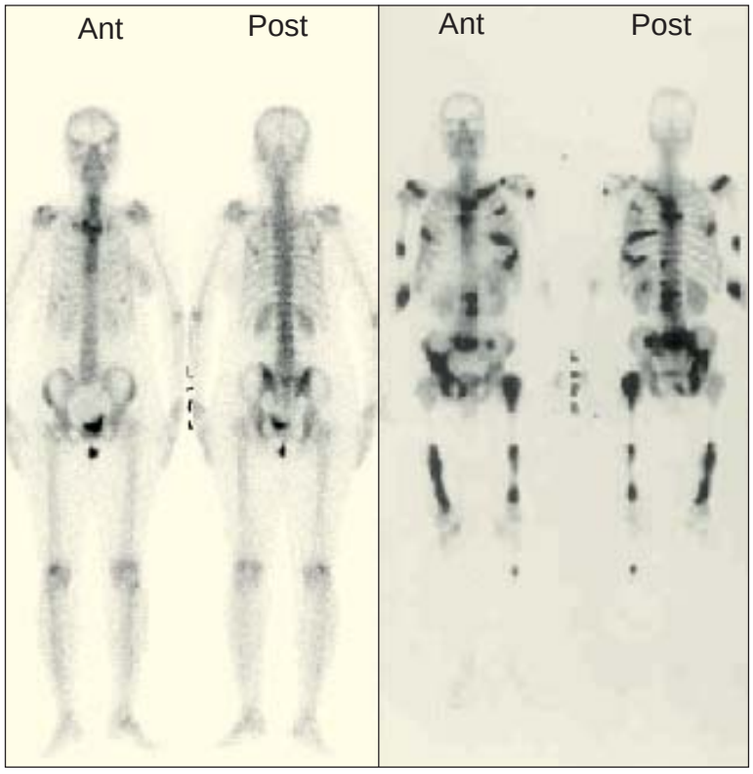
With the use of $^{131}I$ for detection of thyroid abnormalities in early years the field of imaging has come a long way in RMC.

Currently $^{99m}Tc$ is a radio-nuclide of choice in the field of diagnostic nuclear medicine becuase most of the organ specific pharmaceuticals can be tagged with $^{99m}Tc$, for example $^{99m}TcMIBI$, tetrofosmin for myocardial infarction, $^{99m}Tc$ DTPA for renal perfusion, $^{99m}TcDMSA$ for renal cortical defects and $^{99m}TcECD$ for cerebral perfusion etc. Reticuloendothelial cells can be evaluated by injection of $^{99m}Tc$-sulfur which is phagocytosed by these cells and distributed to liver, spleen and bone marrow. GI tract bleeding can be evaluated by tagging RBCs with $^{99m}Tc$. A single injection of bone seeking $^{99m}Tc$ MDP can give image of hot spots in skeleton representing injury or disease of specfic bones.

The recent introduction of Positron Emission Tomography (PET) in RMC is helping detection of lesions in body organs which were not easily amenable to conventional imaging techniques. PET tracers are positron emitting radionuclids such as $^{11}C$,$^{13}N$,$^{15}O$,$^{8}F$ which have a short half life and are produced in the compact cyclotron in RMC. Positron emitted by the nucleus of these labeled compounds is slowed down by interaction with tissue. After annhilation with electron, two $\gamma$ photons of equal energy are produced which travel in opposite direction. PET scanner detects these $\gamma$ photons by a process of coincidence detection and produces image of different organs such as brain, lungs etc.

*Compact medical cyclotron*

PET scan with $^{18}FDG$. *Metabolically active cells take up glucose and appear as dark areas. The whole body can be screened for the spread of cancer. Extensive nodal disease is seen in the left upper chest. No other abnormalities are seen in the PET image of this patient*




*Bone scan with a single injection of $^{99m}Tc$-MDP showing normal bone scan on the left and abnormal (multiple skeletal metastasis) bone scan on the right*

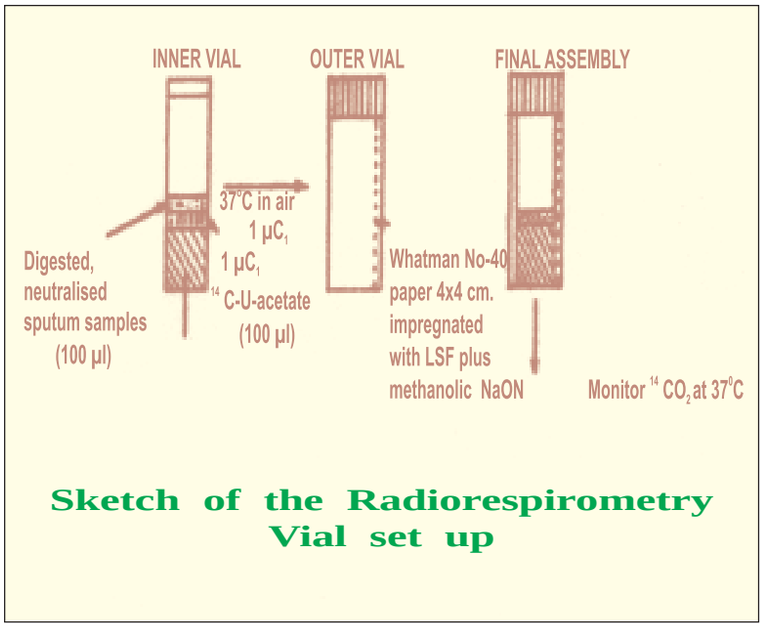
using sequential monitoring of radioaerosols deposited in the trachea. An animal model of restrictive/obstructive lung disease was developed in rabbits using cement as an example of particulate aerosol. Using this model the time taken to develop obstructive disease, the amount of inhaled particles needed to produce disease and several other parameters were studied.

### Techniques for early detection of bacteria

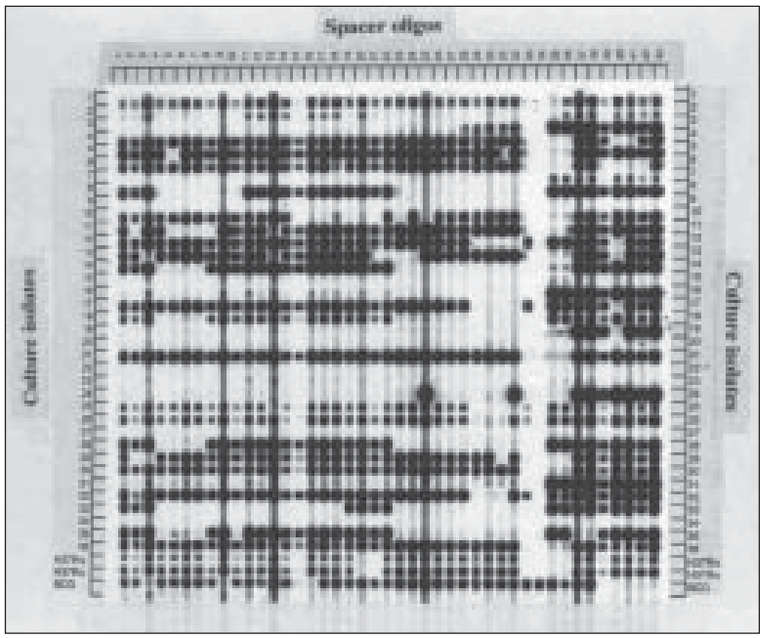
A radiorespirometric technique using $^{14}C$-labeled substrate was developed for early detection of bacteria in biological samples such as urine to determine the presence of urinary tract infection and also to conduct drug sensitivity testing so that an appropriate antibotic could be started. The time for detection and testing of drug susceptibility to tuberculosis was remarkably shortened by this technique from 8-10 weeks to 2-3 weeks.

Development of RIA for detection of antigen for tuberculosis (TB Ag) in cerebrospinal fluid (CSF), serum, pleural and ascitic fluid was initiated in 1978. A few monoclonal antibodies were produced of which the one against the 38kDa antigen was found to be highly specific and useful in RIA and ELISA and also for isolating commercially useful 38kDa antigen. Animal models of tubercular lesions were developed in rabbits (thigh) and mice (brain) in order to establish radioimmunoscintigraphy techniques for detection of tuberculosis lesions. Radiolabeled anti-BCG antibodies were shown to localize in these lesions by radioimmunoscintigraphy and autoradiography techniques.

A recent development in diagnosis of tuberculosis has been the PCR test for the gene coding for a 38 kDA antigen specific to the *M. tuberculosis*. Specific primers, designed at RMC, were found to give satisfactory results. Spoligo-typing is also used for various strains and can throw light on transmission of the disease.




*Radiorespirometry to detect bacilli in sputum. The test is faster than detecting M. tuberculosis by culture and can be adapted for studying drug sensitivity and evaluating potency of drugs*




*Spoligo pattern of M. tuberculosis culture isolates to identify different strains. At the bottom are patterns for H37Rv and BCG strains*

# Cell Biology

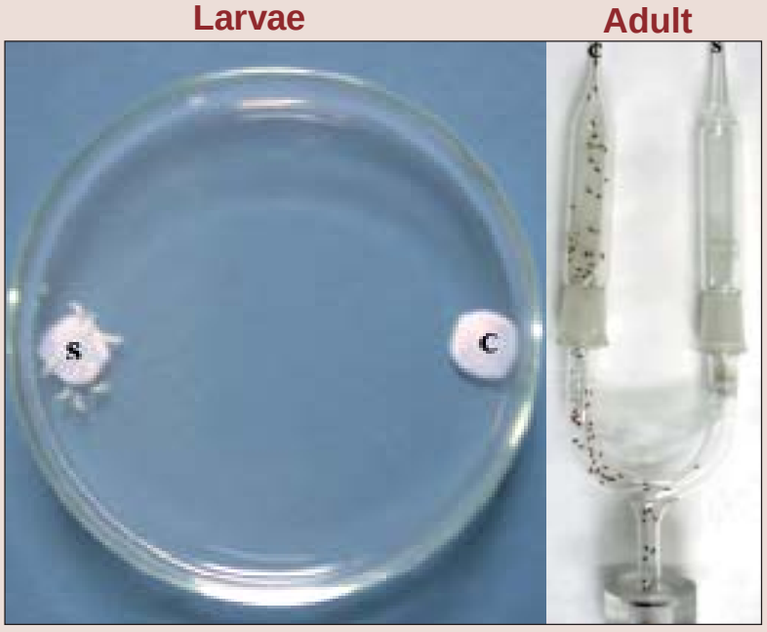
## Link B7

*Cell Biology deals not only with the structure of various types of cells but also with their functions, interactions with neighbouring cells and their responses to different stimuli related to development, differentiation, function and biotic and abiotic stresses. There is a great diversity in living organisms inhabiting earth. There are unicellular or multicellular organisms, prokaryotic or eukaryotic organisms, autotrophic and heterotrophic organisms and so on. Cells of each of these organisms use special molecular mechanisms to respond to these stimuli. In cell biology these responses are quantified at physiological, biochemical or molecular levels. The end result is expression of a certain set of genes intracellularly or on the cell surface. Many of these end products are proteins with specific functions.*

*The central dogma of molecular biology stipulates that in prokaryotes and eukaryotes the information flows from genes (DNA) to messenger RNA to proteins. The present century heralded the establishment of genomics, proteomics, transcriptomics and metabolomics as a major thrust of research in several biological systems of interest. While genomics deals with the structure and organization of the total cellular genome and genomes of chloroplast, mitochondria or plasmids, proteomics refers to the total complement of proteins a cell can make. Transcriptomics deals with the different messenger RNAs produced and finally the vast body of metabolites generated in biochemical reactions in the body contribute to metabolomics. The last few years have seen an avalanche of information in genomics and proteomics due to development of high throughput technologies. The scientific world is currently deeply engrossed in finding out how living organisms make sensible use of this information to conduct the chemistry of their life. An era of functional genomics has begun and the leading edge of research in biological sciences is shifting from mere sequencing of genes and identification of proteins, to investigating their in vivo organization in relation to their function in different tissues and organs. The new discipline on the anvil is system biology, which deals with understanding the functional networking of genes and proteins in the cells and interpreting the molecular mechanisms of the biology of the cell. In order to understand how the various types of living organisms would respond to radiation, it is important to know how normal cells function and how they respond to other stresses. Cells show differences in radiosensitivity and have evolved different mechanisms to repair the damage to their cellular functions. The cells in a particular phase of cell division are more sensitive to radiation than the cells which are not dividing. Research in cell biology is thus a must for any institution involved in biological research.*

# Understanding mechanism of olfactory responses and anaesthesia using Drosophila

*An Inebriometer used for study of general anaesthesia in flies*




*Olfactory responses of larvae and adults can be measured using a simple assay*

# Cell Biology

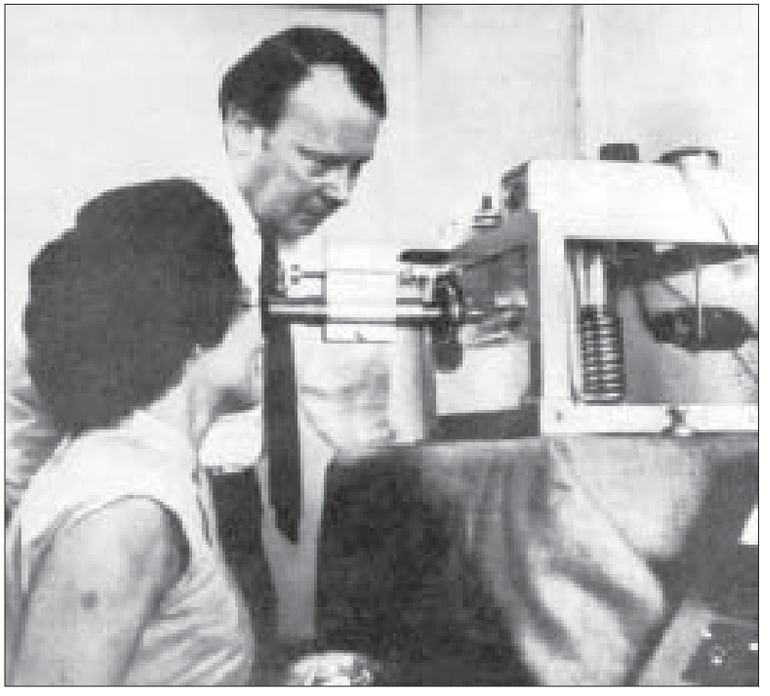
Cell biological research was initiated in DAE institutions to understand effects of ionizing as well as non-ionizing radiation on cellular development and differentiation, intermediary metabolism, vitamin metabolism and mitochondrial bioenergetics using cells from various microbes, plants and mammalian tissues. Immune system being one of the highly radiosensitive systems it was natural that considerable attention was paid to lymphoid cells, which play a pivotal role in immune response. In BARC, this research was extended to assess immunomodulatory effects of a variety of contrivances like antilymphocyte sera, drugs and extracts of medicinal plants and to understand differences between cancer cells and their normal counterparts in terms of surface properties. In another area, neurobiology, scientists at TIFR led by Dr. Obaid Siddiqi initiated research to understand the molecular basis of neuronal responses and neurodegenerative disorders.

## Immunology

The lymphocyte is the pivotal cell in immune response. Surface of lymphocytes has been since long recognized as the site of first immune recognition. Immunisation was shown to bring about discernible changes in the biophysical and immunogenic properties of the lymphocyte surface. Using the simple technique of cell electrophoresis or migration of live cells under the influence of electric field, changes in electrophoretic mobilities of lymph node cells were, for the first time, demonstrated in immune rats. Studies in BARC also demonstrated that Tlymphocytes were more electronegative than B cells.

Treatment of lymphocytes with antilymphocyte serum or antibodies to cell surface markers led to reduction in their surface charged density. Such measurements also revealed that heterologous antisera against immune lymphocytes contained antibodies which reacted against components present exclusively on the surfaces of immune cells. These were anti-idiotypic antibodies. These antisera facilitated immunosuppression of an initiated immune response in antigen specific manner. These studies with antilymphocyte sera also set the stage for subsequent research on immunomodulation using radiations and natural products of plant and bacterial origin.

Medicinal plants are believed to contain several immunomodulatory compounds. In a unique effort, biological activity based purification of an immunomodulatory large molecular weight polysaccharide from the well-known Indian medicinal plant *Tinospora cordifolia* was carried out. This compound was mitogenic to mouse B cells, enhanced antibody production *in vitro* and *in vivo* and also altered secretion of several cytokines by macrophages. Recently, a new prodigiosin like red pigment with significant immunosuppressive as well as antitumour properties has been isolated from an organic solvent tolerant strain of *Serratia marcescens*.



*Prof. E. J. Ambrose of Chester Beatty Institute for Cancer Research London, introducing BARC scientists to cell electrophoresis in early 60's*

In the mid-seventies, several parameters of malignant transformation were being investigated. Agglutinability by some lectins was considered a characteristic of malignant cells. Interaction with the lectin concanavalin A under conditions permitting redistribution of the bound lectin resulted in changes in cell surface charge densities of lymphocytes. This was confirmed using fluorescent labeled lectin. More detailed studies helped establish existence of two sets of receptor (binding)

sites for this lectin on lymphocytes of normal mice. In contrast, their leukaemic counterparts showed presence of only one such type of receptor (binding) sites, which also showed different lateral mobility in the plane of the membrane. These were the first ever reports of this kind of differential behaviour of lectin binding sites. Later, these differences in the characteristics of lectin binding sites could be linked to the maturation status of thymus cells.

Interesting observations were made on enhanced immunogenicity of tumour cells following their treatment with the enzyme neuraminidase, which removed terminal sialic acid moieties from cell surface glycoconjugates. Using experimental tumour models BCG was shown to be a useful adjunct to therapy. A spontaneous tumour lymphosarcoma ascites (LSA) was detected in Swiss mice in BARC and transplantation route related differences in tumourigenicity and immunogenicity were demonstrated. Recent studies showed that tumour-derived cytokine transforming growth factor β regulated tumour growth and also suppressed immunological function.

T cells expressing gamma delta receptors (γδ TCR) form a unique subset of lymphocytes. These cells differ from classical lymphocytes expressing αβ TCR in terms of ontogeny, tissue tropism and gene usage. γδ T cells are gaining considerable importance as antitumour effector cells, which can be exploited for developing immunotherapeutic approaches for cancer. Recent studies at ACTREC demonstrated that Vγ9/Vδ2 subset of γδT cells predominated in the peripheral blood of oral cancer patients and were able to lyse oral tumour cells and tumour cell lines. Heat shock proteins (hsp60/hsp70) expressed on oral tumour cells served as ligands for γδ T cells. These studies further demonstrated sequestration of γδ T cell subset Vδ1 in the tumour compartment.

TCR gene rearrangement studies carried out in the lymphocytes present in the peripheral blood, tumour and metastatic lymph nodes of patients demonstrated a selective clonal expansion of Vγ/Jγ1-3 γδT cells present in the vicinity of the tumour cells. Recent studies also demonstrated stimulation of γδ T cells with non peptidic phosphoantigens such as isopenteylpyrophosphate and bisphosphonates.

In order to investigate reasons underlying immune dysfunctions in oral cancer patients, expression of T cell signaling molecules CD3-ζ chain, CD3-ε, ZAP-70, $p56^{lck}$ were analyzed. Results indicated reduction in signaling molecules in peripheral blood lymphocytes (PBL) of oral cancer patients as compared to healthy individuals with a more pronounced drop in the CD3-ζ chain expression. An inverse correlation of CD3-ζ chain expression as tumour stage progressed from stage I to stage IV was observed. CD3-ζ chain defects can serve as a biomarker for predicting prognosis of the disease and as a measure of response to therapy.

In the late 1970's, a series of discoveries of seminal importance by Tonegawa, Hood and others established that genes for antigen binding proteins were assembled by recombination of several segments of DNA derived from an array of variable(V), diversity(D) and joining(J) and constant(C) regions during lymphoid differentiation. The process is the key to the generation of a large repertoire of antigen specific receptors and antibodies in immune system.

Some of the key components/factors that were likely to participate in V(D)J recombination were isolated and characterized. One of the lymphoid specific enzymes implicated in the repetoire diversification process was terminal deoxynucleotidyl transferase (TdT) which was found to exist in free and nuclear matrix-bound forms in rat thymus nuclei. A megadalton complex (23 S-1.3 megadalton) was isolated from 2-3 weeks old rat thymuses and was shown to contain tightly bound TdT activity. Interestingly, the complex was absent from nuclear extracts of spleen and thymus of adult rats. It was shown to have all the attributes of the postulated recombinase multi-enzyme complex in the pre-B or pre-T cells.

Endocytosis is a process by which living cells take up materials such as nutrients and growth factors from their surroundings. Many viruses, including those responsible for AIDS and influenza, hijack this important process to enter and infect our cells. It is thus of vital importance to study the process in order to understand its underlying molecular details.

The protein dynamin, is an important regulator of endocytosis. Studies at TIFR uncovered the importance of certain phosphorylation events in modulating interactions of dynamin with other proteins. It was found that dynamin was efficiently phosphorylated by kinases of the Dyrk family and that this regulated interaction of dynamin with amphiphysin, Grb2 and Abp1. Over-expression of Sumo in mammalian cells was found to lead to abrogation of dynamin-dependent

endocytosis without affecting dynamin-independent internalization.

## Infectious diseases

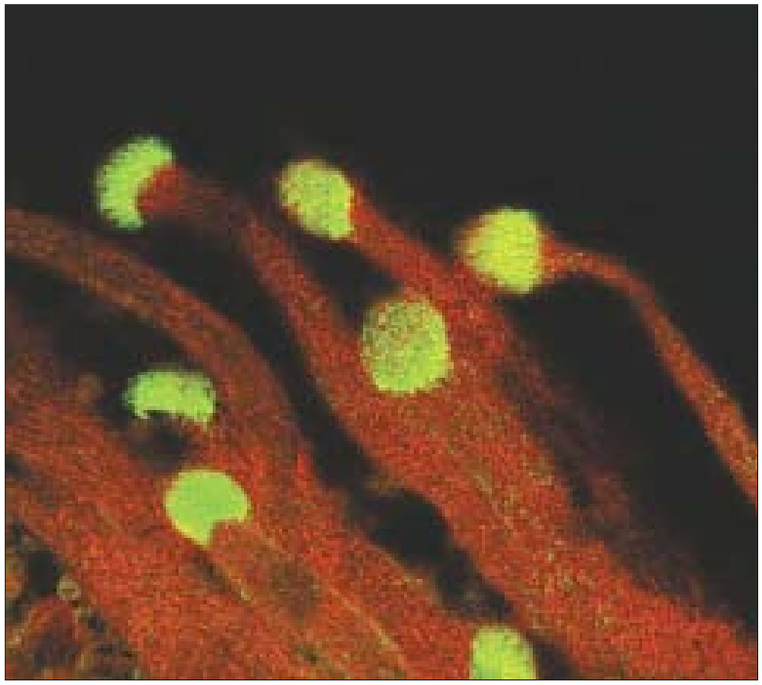
Basic research on leprosy, tuberculosis and malaria has also been supported by DAE. A strain of microorganism (ICRC bacillus) analogous to *Mycobacterium avium intracellulaire* was isolated in Cancer Research Institute from lepromatous patients. It was found to induce strong cellular immune responses in lepromatous patients and contacts. Clinical trials with this vaccine were carried out. A technique for detection of antibodies to phenolic glycolipid-I (PGL-I) of mycobacteria was standardized in RMC. Although antibody to PGL-I alone was not of much use as a predictive test, presence of raised circulating immune complex (CIC) and a negative lepromin reaction appeared to indicate population at high risk.


*Indian isolates of HIV-1 and HIV-2 were cultivated for the first time in CRI. An indigenous sero-diagnostic western blot kit for HIV-1 and HIV-2 has been developed and is now in the*

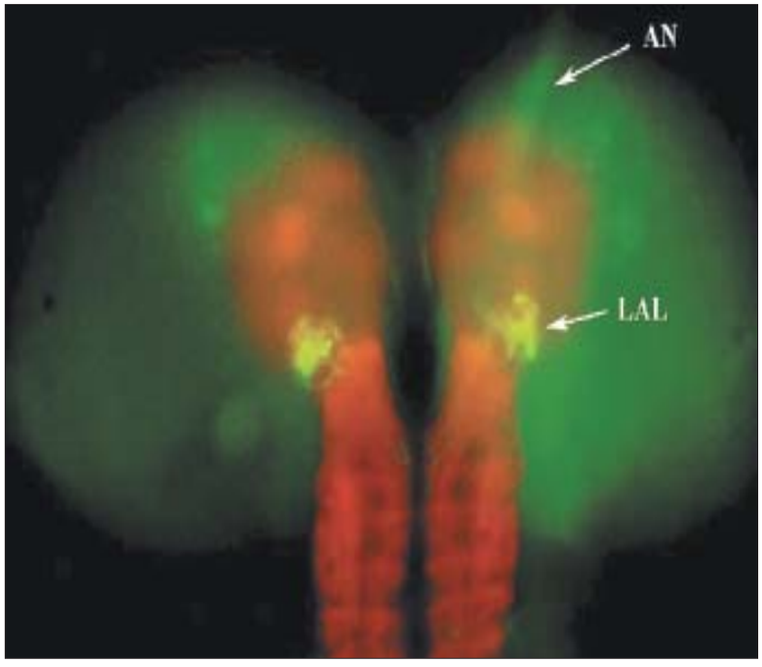
Mechanisms of immunity to malaria are not clearly understood. Attempts are being made to identify antibodies, which play important role in conferring immunity. In order to identify protective antibodies present in immune population, a differential immunoscreen was used to identify several antigenic clones, antibodies against which are present only in immune population and not in malaria patients. Molecular characterization of biological functions and testing antisera raised against these proteins to examine whether parasite growth gets inhibited, is a major research activity in TIFR. As glycolysis is the sole source of energy for *P. falciparum*, one of the causative agents of malaria, studies on molecular characterization of enzymes involved may allow development of novel chemotherapeutic agents. Currently, investigations are being carried out on enolase from *P. falciparum*. Role of post-translational modifications in regulating its biological functions and subcellular localization, are being investigated using a host of spectroscopic (NMR, MS, Fluorescence etc) and biochemical methods.

## Studies related to predisposition to heart ailments

It was shown that an essential pre-requisite to intestinal absorption of dietary folates was hydrolysis of their conjugated polyglutamyl forms to simple monoglutamyl forms by the enzyme folyl conjugase. Abnormalities in plasma lipid, lipoprotein and platelet function can be indicative of predisposition to coronary artery disease. An integrated study was designed to assess the relationship of these risk factors in urban Indian population (of about 200 employees of BARC, the majority being scientists ranging from 30 to 60 years of age). Hypertensive subjects were found to have higher levels of total cholesterol, (TC), triglycerides (TG), VLDL-C, LDL-C and HDL-C. TC/HDL-C ratio was found to be a better measure of the atherogenic index as against individual TC and their fractional lipoprotein cholesterol levels. In diabetic individuals increase in plasma TG, VLDC-C ,TC/HDL-C ratio and platelet aggregation was observed. Significant decrease in HDL-C level was also found. These results are in consonance with the premise that diabetics seem to have higher predisposition to coronary heart disease.



*Localization of the Dynein Light Chain 1 (DLC1) in sperm elongation cones (EC) in Drosophila testis. These structures are formed at the growing ends of spermatids and play a key role in their growth. ER-like membranous network is supported by spectrin cytoskeleton at the EC and Dynein-Dynactin complex control their dynamic reorganization during cell growth. This would have a long-term implication in understanding the ER-Golgi trafficking process, which is a basic cellular activity in eukaryotes*




*Brain of Drosophila larva showing position of the larval olfactory lobe (LAL) and olfactory nerve (AN)*

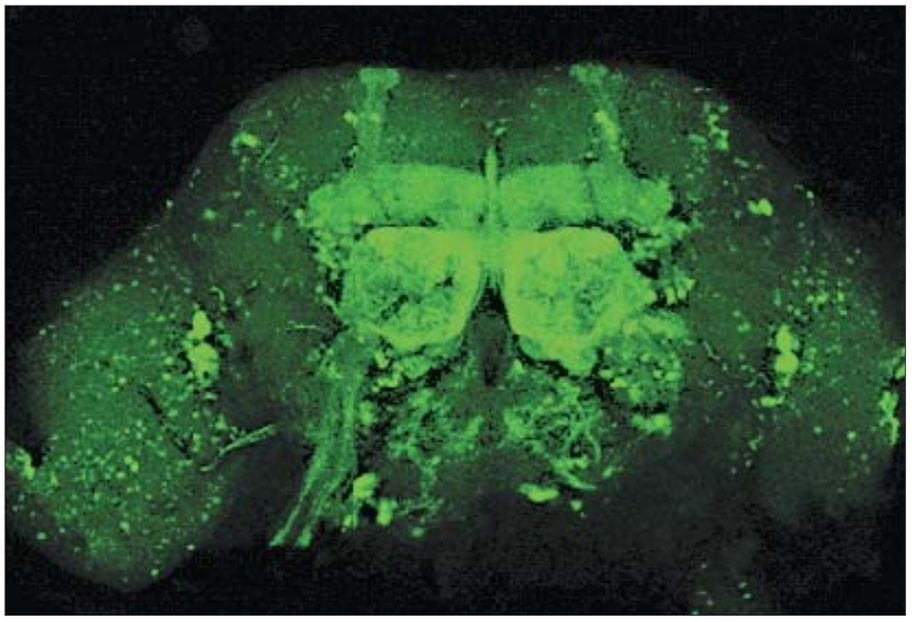
## Neurobiology

After brilliant accomplishments in molecular biology, some of the leading scientists of the world engaged in elucidating the DNA structure and cracking the genetic code decided to turn their attention to a much more complex biological problem-'The Brain'. After a short visit to Benzer's laboratory, Dr. O. Siddiqi, the founder of Molecular Biology in TIFR, decided to use the fly *Drosophila melanogaster*, to understand genetic basis of behaviour. The strategy involved creating flies with defect in behavioural characteristics (behavioural mutants) and it was hoped that analyzing these at the gene level will provide an insight into normal function of the nervous system. A useful phenotype for mutants with defects in functioning of the nervous system is temperature-induced paralysis. Some of these were used by other scientists worldwide to unravel the functioning of nerves and synapses. Interesting mutants that were unable to smell and taste were created to understand the olfactory behavior. Studies were initiated to understand how the neuronal circuits which process smell signals, develop and also the mechanism by which sensory neurons are specified and how they develop and grow to form functional connections. The olfactory system provides many distinct advantages for the study of cell fate specification, neuronal connectivity and pattern formation within the central nervous system.

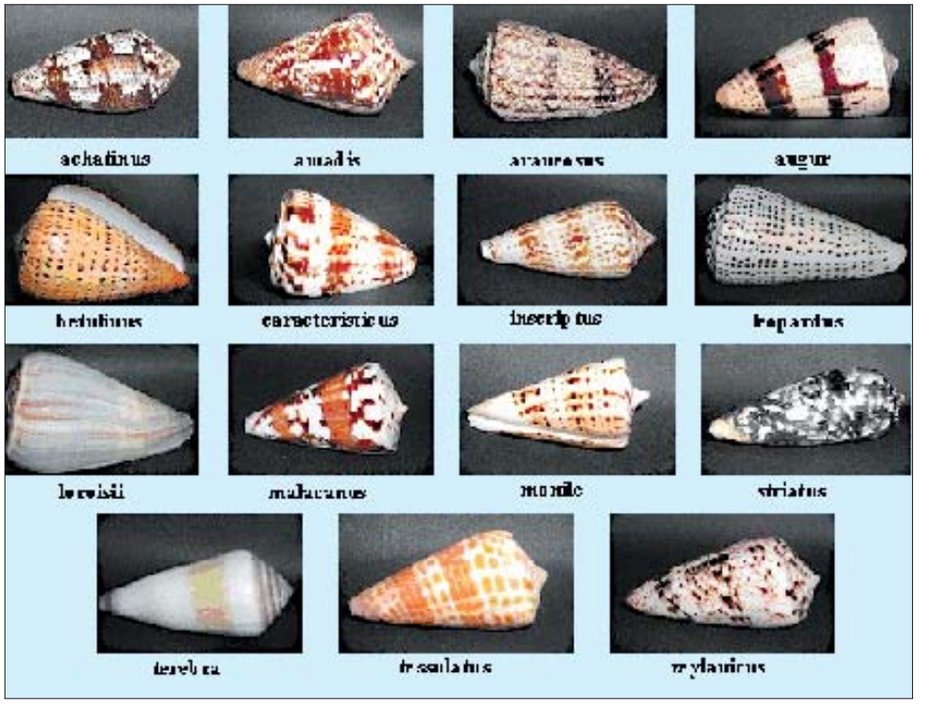
In higher organisms, information processing occurs through specific neuronal networks. Understanding of molecular events that occur at inter-neuron junctions (synapse) is critical for elucidation of higher brain functions. Intensive research is being carried out to understand molecular mechanisms involved in release and uptake of neurotransmitters at synapses using genetic, electrophysiological, immunological and pharmacological approaches to delineate macromolecules involved and temporal sequence in which they perform their function. To date there is very little molecular information available about how animals respond reversibly to anaesthetics. Molecular mechanisms involved in anaesthesia are being investigated using genetic tools by creating mutants of Drosophila, which are resistant to inhalation of anesthestic like halothane. Molecular analysis of genes identified in such

screens will throw valuable light on mechanisms underlying anaesthesia.

Neuronal cells are polarised with the synapse located at one end of the cell. This raises the question of how molecules are transported from the cell body where they are synthesised to the synaptic end. Using Drosophila genetics coupled with imaging and cell biological methods attempts are being made to understand mechanisms underlying different processes in the nervous system and also on specific motor molecules involved in transporting various kinds of molecular cargo across the long axons. These studies are being extended to mammalian brain development and stress induced changes in brain. Among other things, mechanisms that control cell fate in the mammalian cerebral cortex are being investigated. Cerebral cortex, the site of all higher cognitive and perceptual functions, is divided into functionally specialized areas containing distinct types of neurons. How is this diversity of neurons generated in development? What mechanisms regulate this process? Techniques in molecular biology, such as retroviral gene transfer, tissue culture, immuno-histochemistry, *in situ* hybridization, and neuroanatomy are being used to address these questions. Molecular mechanisms involved in damaging effects of prolonged and severe stress on adult mammalian brain, which may result in psychiatric disorders e.g. depression, are also being investigated. Such pathology has been linked to decreased neurogenesis in hippocampus. The focus is to understand pathways that contribute to regulation of basal hippocampal neurogenesis, and may be recruited by stress and antidepressant treatments to regulate structural plasticity.



*Adult Brain of Drosophila melanogaster. The olfactory lobes and the mushroom bodies are visualized by expression of Green Fluorescent Protein*




*A selection of Conidae from Indian coasts being investiagted for peptides of therapeutic value*

## Studies on neurodegenerative disorders

At SINP and BARC research has been initiated on certain neurodegenerative disorders that include Huntington's disease (HD), dentatorubral pallidolysian atrophy, spinal bulbar muscular atrophy and several subtypes of autosomal dominant spinocerebellar ataxias (SCA1, SCA2, SCA3, SCA6, SCA7, SCA8, SCA10, SCA12, SCA17). All these diseases are progressive and ultimately fatal disorders that typically begin at middle age with a wide range of variation in the age at onset. Rate of disease progression is also highly variable. Expansion of polymorphic glutamine (CAG/CTG) repeats in specific

proteins is implicated in a number of neurodegenerative diseases where loss of neurons is the primary consequence of such an expansion.

In most of the diseases, the age at onset roughly correlated inversely with the number of glutamine repeats in the protein. Various studies indicated that number of glutamines in the repeats is the most important determinant but does not explain all the variations. Given the immensely complex biochemical events that are thought to occur due to glutamine repeat expansion involving a large number of proteins including caspases, chaperons, and poly-glutamine interacting proteins and defects in the degradation of the mis-folded/aggregated proteins, it is likely that a small variation (as SNPs) in any one of the these genes would modify the progression and age at onset of these diseases. The 3D structure determination of the poly-Q interacting proteins together with the detection of the variation in the genes involved in apoptosis would be expected to delineate the pathways involved in neurodegeneration and provide possible therapeutic intervention.

## Developmental biology

In TIFR, the molecular biology work was extended to more complex problems such as developmental genetics in *Caenorhabditis elegans* to study the genes involved in cell fate determination during development. It is noteworthy that this worm possesses only 959 somatic nuclei, making studies on the cell lineage of this organism easily tractable. The genetic mechanisms that control the formation of the segmented body plan in the fruitfly *Drosophila melanogaster* are being investigated. Some of the genes involved in this process responsible for wingless and the bithorax complex have been shown to play important roles in a variety of cellular processes including those in humans.

The interest in developmental mechanisms extended to plants using the model system of the moss *Funaria hygrometrica* to establish how the choice between two distinct cell lineages chloronema and caulonema takes place. For the first time, it was established that plant hormones such as indole acetic acid, cyclic AMP, cell density and pH played crucial role in moss differentiation.

### Programmed cell death in bacteria

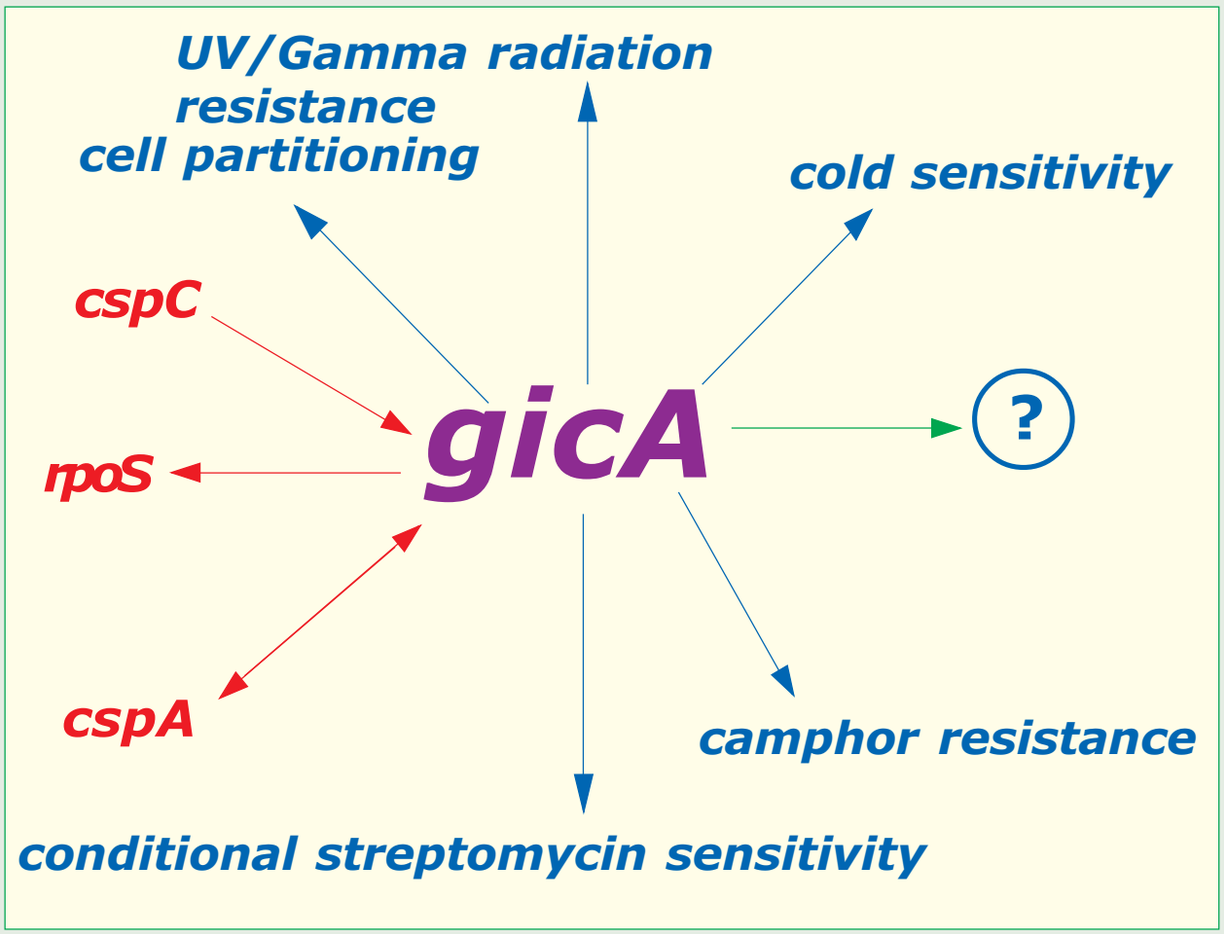
A molecular mechanism of programmed cell death (PCD) that is similar to that found in eukaryotes was reported in the prokaryote *Xanthomonas campestris* pv. *glycines* AM2 (XcgAM2), the causal agent of bacterial pustule disease of soybean (*Glycine max*). Xcg was found to undergo post-exponential PCD during certain nutritional conditions. Other *Xanthomonas* strains showed similar PCD process. The cells display a typical exponential growth phase when cultured in minimal medium. However, in a protein rich medium, instead of eventually entering the normal stationary phase, the culture was found to undergo extensive PCD. The process was arrested when starch was added to the medium. While undergoing the PCD the organism was found to undergo morphological changes to transform into spherical membraneous apoptotic bodies. It also produced a caspase-3 like protein which displayed strong binding to human caspase-3 antibody in a Western blot. The changes in plasma membrane were evident from the binding of transforming cells to annexin V. PCD and caspase negative mutants could be obtained by treating wild type Xcg cells with N-methyl-N'-nitro-N-nitroso guanidine (MNNG). The caspase negative mutants were found to retain the other traits of the wild-type strain. However, the caspase mutation resulted in small colony morphology.

*Western blot using human caspase-3 antibody showing presence of caspase-3 in Xanthomonas strains*

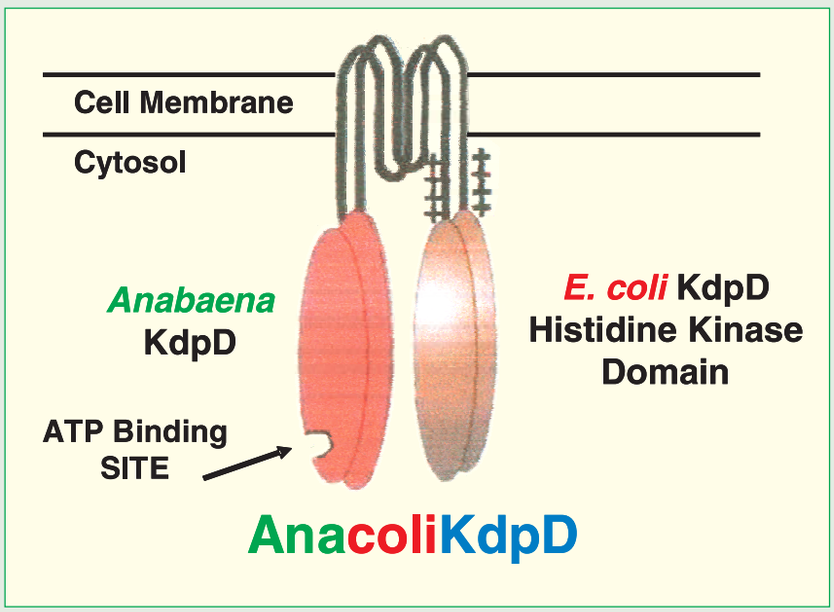
# Molecular Biology and Biochemistry

## Link B8

*Gregor Mendel illustrated the basic pattern of inheritance and suggested possible underlying mechanisms. He achieved this without any information on the chemical composition of genes or behavior of chromosomes. He also had no inkling of how genes are copied between generations, how genes work or how genotypes produce phenotypes. The elucidation of the structure of DNA, the molecule of heredity, indeed life itself, by Watson and Crick in 1953 is one of the greatest triumphs of twentieth-century biology. With this discovery a new discipline called Molecular Biology evolved at the interface of Biology, Chemistry and Physics. Geneticists, biochemists, biophysicists and molecular biologists have contributed to our current understanding of molecular basis of life. There are many steps from genotype to phenotype. Genes by themselves cannot produce phenotypic result such as color of eye, skin or flowers, height of plants or humans etc. Proteins are the real work-horses in the cells. The information for producing a specific protein is present in DNA, which is then copied to RNA that subsequently dictates protein synthesis. Over the years, molecular details of the processes of transfer of information from DNA to RNA to Protein, known popularly as Central Dogma, have been elucidated. Today, molecular biology is a core discipline. It has provided foundations of industry oriented disciplines of genetic engineering and biotechnology. The vast knowledge of how genes function has arisen from the ability to manipulate genomic structure, create transgenic organisms harboring genes originally from other organisms and selectively knock out expression of desired genes. Microbes are the most easy to manipulate. Yet today, even higher organisms are not beyond the reach of this technology.*




*A model depicting gicA a gene that regulates E.coli growth in cold as a global regulator. Red arrows indicate regulation of a gene. Blue and green arrows indicate the phenotypes*




*Genetic engineering of a novel chimeric stress sensor*

# Molecular Biology and Biochemistry

Dr Homi Bhabha realized in the early sixties that a programme in Molecular Biology should be undertaken by DAE. This thought got further impetus with the series of lectures by Walter Rosenblith, the American biophysicist, who was among the galaxy of celebrity scientists, including seven Nobel laureates, that attended the formal inauguration of TIFR in 1961. Soon thereafter, Homi Bhabha, at the suggestion of Leo Szilard, decided to invite Obaid Siddiqi to set up such a programme at TIFR.

Initially, the basic research in molecular biology in DAE was centered on genetic and molecular studies on microorganisms like *E. coli*, and *yeast*. As the principles of molecular mechanisms for information flow from genes, such as one-gene-one-polypeptide hypothesis, DNA structure, base pairing rules, RNA structure, semi-conservative replication, translation, transcription, transformation and DNA recombination got established, research groups at TIFR started enquiring into the mysteries of life at one or the other macromolecular level. Investigations in Siddiqi's lab at this time led to new insights into the molecular mechanisms of DNA recombination and expression of foreign DNA in bacterial cells. The enthusiasm for basic research in molecular biology at TIFR was also shared by scientists in the Bio-Medical Group of BARC leading to establishment of teams working on principles of genetics and biochemistry to gain a mechanistic understanding of basic biological processes.

The first molecular biology group at AEET, as BARC was then known, was set up in 1963. Though the number of molecular biologists here was never very large, by early 1980s, some of the contributions made by BARC scientists were nationally and internationally recognized. The bacterium *Haemophilus influenzae* was a naturally transforming organism and hence was a model system to understand DNA biology. Using heavy water it was shown that genetic transformation in *H. influenzae* was affected by displacement of the homologous resident DNA strand by a single segment of donor DNA, which underwent fragmentation after entry into cells. Several mutants of *H. influenzae* defective in DNA repair and recombination were isolated. Studies on recombination defective mutants (rec mutants) in *E. coli* led to understanding of differences between RecBCD and RecF pathways of general recombination. These pathways were shown to differ in the nature of recombinant molecules they produced and in the kinetics of the recombination products. The RecBCD pathway integrated largely double stranded DNA while the RecF pathway integrated long stretches of single stranded donor DNA. While explaining molecular mechanisms of these differences, it was proposed that RecBCD enzyme had an important role in the synthesis of the complement to single strand of donor DNA, transferred during *E.coli* conjugation. This was unexpected from the known properties of purified RecBCD enzyme but was consistent with several unexplained properties of *recB recC* mutants. Evidence obtained subsequently indicated that *in vivo* RecBCD was complexed with several other proteins including RecA, PolA and DNA ligase which inhibited its nuclease activity and might account for its role in DNA synthesis. Role of various genes of the RecBCD and RecF pathways in DNA amplification was also examined using transposons Tn*9* and Tn*1721*. Several new genes related to cold sensitivity, streptomycin resistance and carbohydrate uptake etc. were isolated, mapped and their structural and functional aspects were characterized in *E.coli*. Significant results emerging from analysis of these genes included identification of a common bacterial regulator of cold sensitivity and sensitivity to UV and ionizing radiations. These also helped in identification of novel regulators of cAMP synthesis in *E. coli.* Monitoring kinetics of expression of the cold stress gene *cspA* following shifting of *E. coli* cells to cold provided significant clues to the regulatory cascade that enables cells to transit from the initial lag period to exponential growth.

Two *H. influenzae* UV repair genes, one of which showed partial homology with a human cancer gene, were also characterized. With the availability of good expression vectors molecular analysis of genes was extended to structural studies of their protein products. Two non-specific acid phosphatases of *Salmonella enterica* sv *typhimurium* (the class A enzyme PhoN and the class B enzyme AphA), which have considerable potential in bioremediation of nuclear waste, were purified and crystallized and their structures determined by X-ray diffraction. Human carbonic anhydrase I gene was also cloned under an inducible promoter in *E. coli* and subjected to site specific

mutagenesis. Characterization of partially purified products of six mutants gave important insights into the catalytic mechanism of this enzyme associated with important human diseases like glaucoma. A *cry2*-type gene (cry2Aa4) from *Bacillus thuringiensis* was cloned and sequenced. Determination of the insect host range of the recombinant protein and sequence alignment with other cry proteins helped in confirming the domains that specify toxicity against dipteran and lepidopteran insects.

### Analysis of a case of somatic instability in maize from India

*Expression and segregation of somatic instability of a C-I$^{m}$ case*

A case of somatic instability affecting aleuron colour in a strain of maize from India with flint backgorung was analysed. The somatic instability was localized to the *C*$^{1}$ (Inhibitor allele of *C ) locus* on the short arm of chromosome 9. Molecular studies indicated involvement of transposable element in instablity.

With the advent of the era of genomics, based primarily on high throughput automated sequencing and *in silico* analysis of sequences, several programmes were initiated in the area. The presence of high-powered computer facilities at BARC were very helpful in this endeavour. An in-house software written to scan the genome for one or two base variants of short sequence motifs led to the proposal of a mechanism of adaptive mutagenesis.

## Molecular biology of nitrogen fixation

Nitrogen is a crucial constituent of all proteins. Though it is abundant in the atmosphere plants are unable to assimilate nitrogen as long as it is in the free elemental form. However, they can take it up when it becomes available in the bound form as a nitrogenous compound. Certain microorganisms and single celled organisms like bluegreen algae could help in this direction. Studies on genetic regulation of nitrogen fixation were, therefore, actively pursued.

The heterocystous *Anabaena* possesses a discontinuous *nifK…DH,* which rearranges to a contiguous *nifKDH* during heterocyst differentiation. In contrast, the non-heterocystous *Plectonema* possesses a contiguous *nifKDH* operon and lacks DNA elements and genes involved in gene rearrangement. A small, endogenous 1.45 kb plasmid from non-heterocystous, filamentous cyanobacterium *Plectonema boryanum*, was characterized and cloned into pBR 322. Restriction mapping was done to enable its use in development of cyanobacterial cloning vectors. A shuttle vector for this cyanobacterium was developed. Role of *ntr* genes in *nif* gene expression in *Anabaena* and *Klebsiella* was investigated. *Plectonema boryanum,* a microaerobic diazotroph, showed reciprocal alternating cycles of nitrogen fixation (N-phase) and photosynthesis (P-phase). Molecular studies on the regulation of nitrogen fixation and photosynthesis have shown that expression of some of the important photosynthetic genes was decreased while that of *nif*H was increased when cells moved from P-phase to N-phase. Though the level of CP47 remained unchanged, the assembly in thylakoid was defective in N-phase. The studies showed that PSII and PSI were uncoupled during N-phase and nitrogen fixation required only PSI activity.

Regulation of expression of *nif* and *hut* operon in *Klebsieilla pneumoniae* by *gln*A linked genes of *E.coli* was examined. The

results indicate that *gln*A product is not mandatory either for derepression or repression of the *nif* and *hut* operons. But *gln*A does seem to influence their expression. This is because deletion of an approximately 300 bp region within *gln*A renders these operons constitutive.

## Molecular biology of stress

Studies to understand the cellular and molecular basis of responses to major agricultural stresses (salinity, drought, heat, nutrient deficiency etc) were initiated using nitrogen-fixing cyanobacterial strains of *Anabaena* as model systems. Using radiotracer $^{22}Na$, $Na^+$ transport in cyanobacteria was characterized and it was shown that curtailment of $Na^+$ influx was the major mechanism of halotolerance in *Anabaena* strains. It was further shown that inherent salt tolerance of strains could be upgraded by modulating $Na^+$ influx exogenously by external pH, nitrogen source and $K^+$ levels. Sensitivity of cyanobacterial nitrogen fixation to salinity was shown to be due to repression of *nifKDH* expression and diversion of cellular energy from $N_2$ fixation to $Na^+$ efflux and other osmoregulatory proceses. Similarly, using [$^{35}$S]methionine radiolabeling *in vivo*, distinct salinity and osmotic stress-regulated differential gene expression was shown in cyanobacteria.

In *Anabaena* a strong correlation between the presence/absence of a large number of osmotic stress-induced proteins and osmotolerance/sensitivity respectively was established for the first time. A subtractive RNA hybridization technique was developed to screen the genomic library and clone a large number of osmoresponsive genes from a marine cyanobacterium *Anabaena torulosa*.

Currently, cyanobacterial heat-shock response and response to potassium deficiency are also under investigation in detail. Although heat-shock proteins (HSPs) of *Anabaena* appear to resemble those of other bacteria, kinetics and regulation of their expression are quite different. An *Anabaena* strain was shown to exhibit superior thermotolerance than *E. coli*, on account of prolonged synthesis, greater stability and accumulation of Hsp60 proteins in cyanobacterium. Two *hsp60*-like genes, *groESL* and *cpn60*, were cloned from *Anabaena* and shown to express differently under different environmental stress conditions.

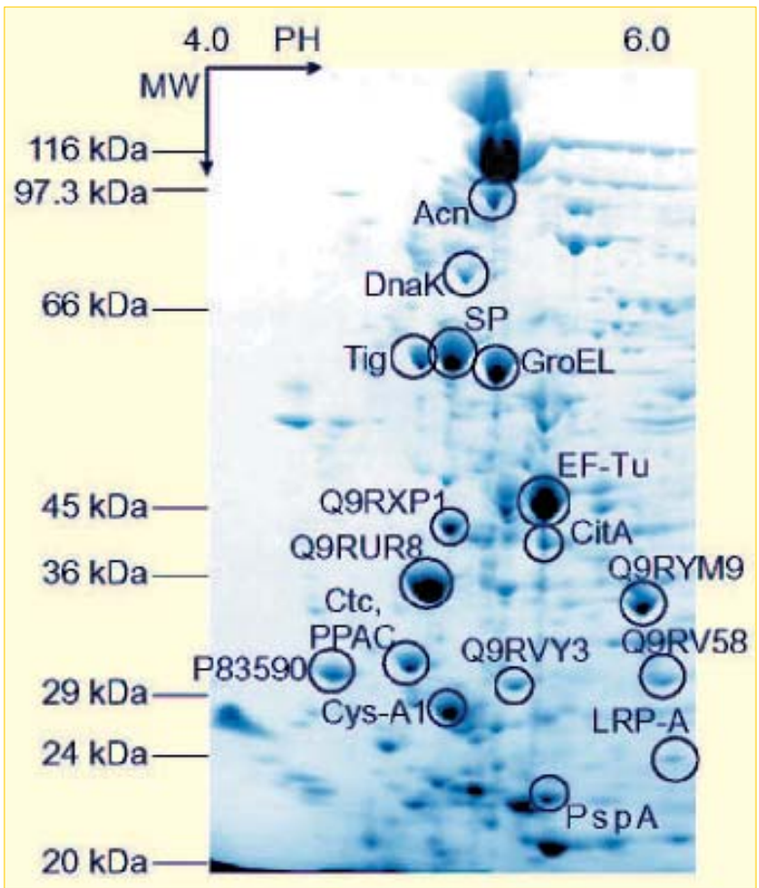
Potassium deficiency was shown to result in multiple defects in cyanobacterial growth and metabolism. In particular, potassium starvation induces expression of several novel potassium deficiency proteins (PDPs), which include photosystem constituents, oxidative stress alleviators, chaperones and the KdpATPase. The major *kdpABGCD* operon encoding *Anabaena* KdpATPase was cloned and sequenced to reveal many unusual features. In particular, the likely potassium signal sensor (KdpD) is found to be a truncated protein while the *kdp*E gene, which encodes the transcriptional activator of *kdp* operons in bacteria, is absent. A chimeric Anacoli*kdpD* was constructed and shown to function in *E. coli*. The role of *kdp* signaling system in regulation of KdpATPase and other PDPs is under investigation.

Analysis of stress proteins induced by salinity and drought stresses in strains of several agriculturally important bacteria such as *Anabaena, Rhizobium, Psuedomonas* and rice cultivar Bura Rata, was carried out. Six major salt stress-induced LEA proteins were recently identified using proteomic approaches and shown to (a) accumulate during exposure to salt stress, (b) lead to growth slowdown, and (c) be degraded during recovery from salinity stress in the salt tolerant rice cultivar Bura Rata. Proteins involved in the biodegradation of γ-hexachloro-cyclohexane or lindane, a common pesticide, in a *Sphingo-monas paucimobilis* strain were identified. These have potential application in bio-remediation of pesticide-contaminated soils.

## Low temperature tolerance in groundnut

Groundnut is grown in tropical and warmer temperate regions throughout the world. Groundnut plants show maximum growth at 28$^0$C, but experience severe metabolic perturbances when exposed to temperatures below 12$^o$C. However, Trombay groundnut variety, TAG-24 is able to survive at 12$^o$C for several days without apparent injury. TAG-24 recovers rapidly from the stress and resumes normal growth at its optimal growth temperature. In order to understand the basis for this tolerance, low temperature induced secretion of proteins in leaf apoplast, a widespread phenomenon, was studied. Anti-freeze proteins in winter and spring rye, winter and spring wheat, winter barley and spring oats are secreted in the cold acclimated plants. Cold induced apoplastic protein (AHCSP33-*Arachis hypogea* cold shock protein 33kD) from TAG-24 was isolated and


*Colonies of Deinococcus radiodurans on TGY agar plate*




*Proteomic analysis of Deinococcus radiodurans. Circled proteins were identified by N-terminal amino acid sequencing*

characterized. AHCSP33 showed homology to thaumatin-like (TL) protein family and had antifreeze activity. Like several TL proteins AHCSP33 is also targeted to the apoplast and persists for several days after low temperature treatment. It possessed intra-chain disulphide bonds which is a well conserved feature of TL proteins.

### Molecular biology of stress due to UV

Ionizing radiations induce DNA damage in *E. coli,* as in many other organisms. Detailed study of the nature of the damage and mechanisms leading to that damage revealed that structural integrity of the membrane was important for rejoining radiation-induced single strand breaks and *de novo* RNA synthesis is a pre-requisite for Type III repair in irradiated *E. coli* cells. The photoreactivating enzyme encoded by *phrA* gene, was purified from *E. coli* and its RNA cofactor was shown to be involved in recognition of UV-induced dimers. UV-induced mutagenesis and effects of UV on the biology of nitrogen-fixing *Anabaena* strains was also investigated.

### Molecular mechanisms of resistance to ionizing radiation

A bacterial species of *Deinococcus,* discovered at Trombay, was found to be resistant to extremely high doses of radiation. Studies aimed at unraveling the molecular mechanisms underlying this characteristic led to (a) identification of several radiation-responsive genes by proteomic approaches, b) cloning and characterization of known DNA repair genes (like single stranded DNA-binding protein gene, *ssb*) which have unusual or aberrant structure/function in *Deinococcus*, and (c) identification of a ROS-scavenging role for pyrroloquinoline quinone (PQQ) and improvement in the oxidative stress tolerance of *E. coli* by transfer of *pqq* synthase gene from *Deinococcus radiodurans*.

### Extremely halophilic archaebacteria

Some microbes are known to survive under extreme conditions of temperature, pH, salt etc where normal organisms will not survive. They are termed as extremophiles. Biologists have been always curious to find out how enzymes in these organisms work under such extreme conditions. The extremely halophilic archaebacteria *per force* require very high concentrations of salts of like sodium chloride and potassium

chloride (higher than 20% NaCl) to survive. These organisms have developed mechanisms for overcoming the external osmotic pressure by an intracellular high concentration of KCl (>2M). Thus the cell machinery and enzymes of halobacteria are adapted to high intracellular salt concentration. The enzymes of carbohydrate metabolism were purified and their structure–function relationship was investigated. These studies showed the presence of a modified Entner-Doudoroff pathway for utilization of sugars and absence of phospho-fructokinase (PFK), a key glycolytic enzyme. Currently, studies are underway to understand stress responses and repair mechanisms, including DNA repair, in these organisms.

## Bioinformatics and biotechnology

Bioinformatics is an emerging discipline. A marriage of biotechnology with information technology yields very large and swift information processing capabilities. These have great potential in almost every branch of life sciences. Naturally, pro-active steps towards acquiring such capabilities were taken. A beginning was made in late 1980's with computer analyses of phylogeny by hierarchical clustering of published nucleotide sequences of *nifH* genes from 27 different genera. These were found to be in agreement with the accepted taxonomy and predicted likely paths of evolution within diazotrophs. Codon usage analysis was carried out to identify ORF's most suitable for expression in *E. coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae*. With the installation of an automated DNA sequencer genomics and the *in silico* analysis of genes, vectors and even whole genomes of bacteria can be done. Current interests range from molecular taxonomy using 16S rRNA gene-based bacterial identification, stress responsive genes from different organisms to analysis of molecular (DNA) markers of crop plants and of single nucleotide polymorphisms in breast and retinal cancer samples.

Efforts were also made to seek biotechnological applications using bacteria. Noteworthy among these have been isolation of genetically distinct *Bacillus* strains for control of insect pests and biodegradation of pesticides. *Anabaena* strains are being engineered for use as N/P/K biofertilizers.

Other studies which used molecular techniques were: isolation of a rice gene involved in meiotic recombination, site-directed mutagenesis in malic enzymes genes, use of microsatellites in analysis of Down's syndrome and population genotyping in humans, DNA based diagnosis of diseases like DMD, leptospirosis and a rare thyroid cancer MEN2B etc.

## Enzymology of DNA repair and recombination

DNA being the important target of radiation damage, several studies are currently underway to understand the mechanism of DNA repair. Defects in DNA repair result in genomic instability with grave consequences. Efforts are, therefore, focused towards understanding physico-chemical nature of genome repair/recombinational events, which are carried out by an ensemble of proteins, now called as "PROTEIN MACHINES". In this effort, the following protein-machines have been under study: Homologous Recombination-(*E.coli* RecA, Human Rad51 and Human Rad52); Mismatch Repair-(*E.coli* MutS, MutL, MutH, UvrD-helicase, DNA-polymerase); Chromosome translocation proteins-(Human Translin, Human Trax, DNA-ligase). The findings point out some novel aspects of RecA mediated DNA motor activity as well as MutS protein treadmilling on mismatched DNA. Recently, *Chlamydomonas reinhardtii,* a unicellular eukaryotic photoautotroph has been developed as an efficient paradigm for forward genetics that receives transgenes by agrobacterial mode to study the linkage between programmed cell death and genome repair.

## DNA repair and replication

The major regulatory element of DNA replication in bacteria is the process that controls the initiation of DNA replication. This is because the rate of cell division is governed by the frequency of initiation of DNA replication. Studies revealed that the DNA replication is intimately associated with cell membranes in bacteria and several enzymes involved in DNA metabolism are associated as a multienzyme complex. It was found that the DNA replication units consisting of DNA polymerase and other ancillary enzymes such as ribonucleotide reductase, nucleotide kinase etc are present in DNA membrane complex. Assembling and disassembling of these units may be directly related to DNA synthetic needs of cells. Structural integrity of the cell membrane was also found to be essential for the repair of DNA lesions arising from UV and ionizing radiation. Besides, cellular RNA and protein synthesis were

## Smart moves by living systems to repair the damaged DNA

• **Photo-reactivation:** The damage caused by UV exposure is repaired by photo reactivation. The DNA photolyase binds to thymine dimers (T-T) in dark. When light shines on the cell, folic acid uses this energy to cause conformational changes in the photo lyase-DNA complex, which breaks the bond between T-T dimmer.

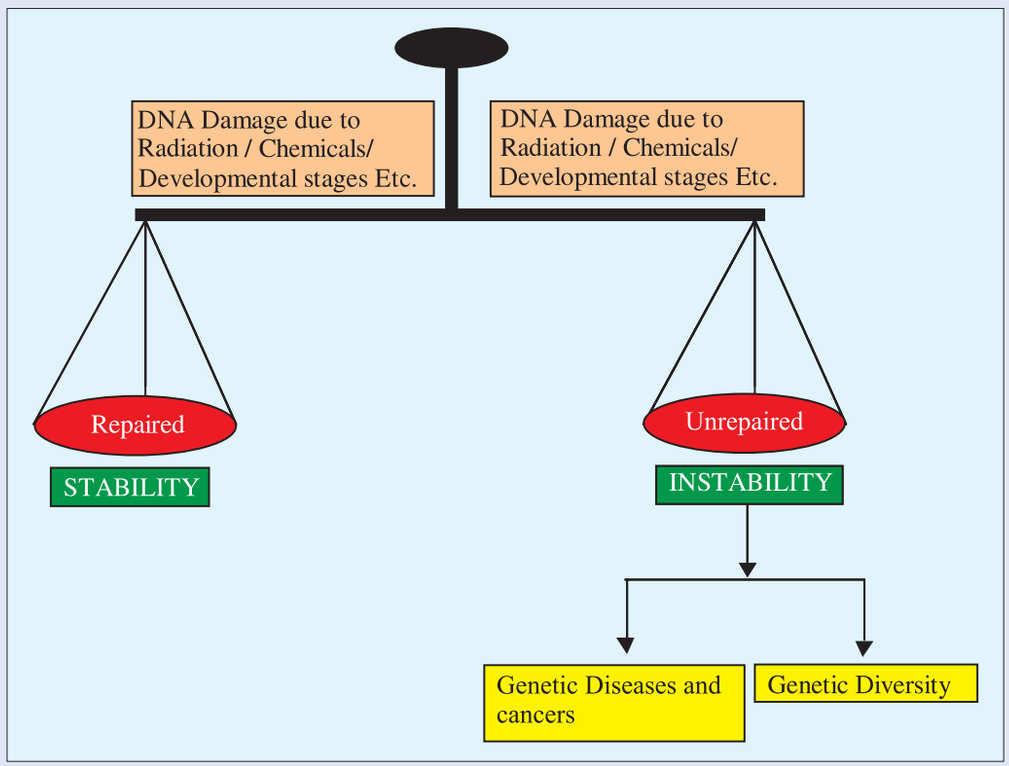
• **Nucleotide excision repair (NER):** If the nucleotide is damaged it is excised and repaired by nucleotide excision repair (NER). In this process an oligonucleotide is removed from the damaged strand and the gap is filled by a special set of enzymes, whose job is to detect the damaged base, remove it and join the strands together.

• **Base excision repair (BER):** Sometimes a base may be lost resulting in apurinic and apyrimidinic sites. A special enzyme, Uracil-DNA glycosylase recognizes and removes U in DNA. This is called Base Excision repair in which AP endonuclease makes a nick at the 5' and 3' side of the AP site. Exonuclease removes the short region of DNA and Pol I and ligase fill the gaps.

• **Mismatch match repair (MMR)** DNA replication is essential in all life forms. Many times errors are introduced in DNA sequence during replication, which are recognized by mismatch match repair system (*mutL*, *mutS* and *mutH* gene products), which detects the differences in the methylation patterns of newly synthesized strand and the old strand.

• **Post-replicative recombinational repair:** Exposure to ionizing radiation causes single strand breaks (SSB) in DNA. If the thymine dimers and base modifications are not repaired, the following replication cycle will also result in a gap opposite the damage. These types of damages are repaired by post replicative, recombinational repair, which uses the sequence information from the homologous DNA strand. This type of repair closely resembles the homologous recombination, which is mediated by RecA and other proteins like SSB, RuvA, RuvB, RuvC or their homologues etc.

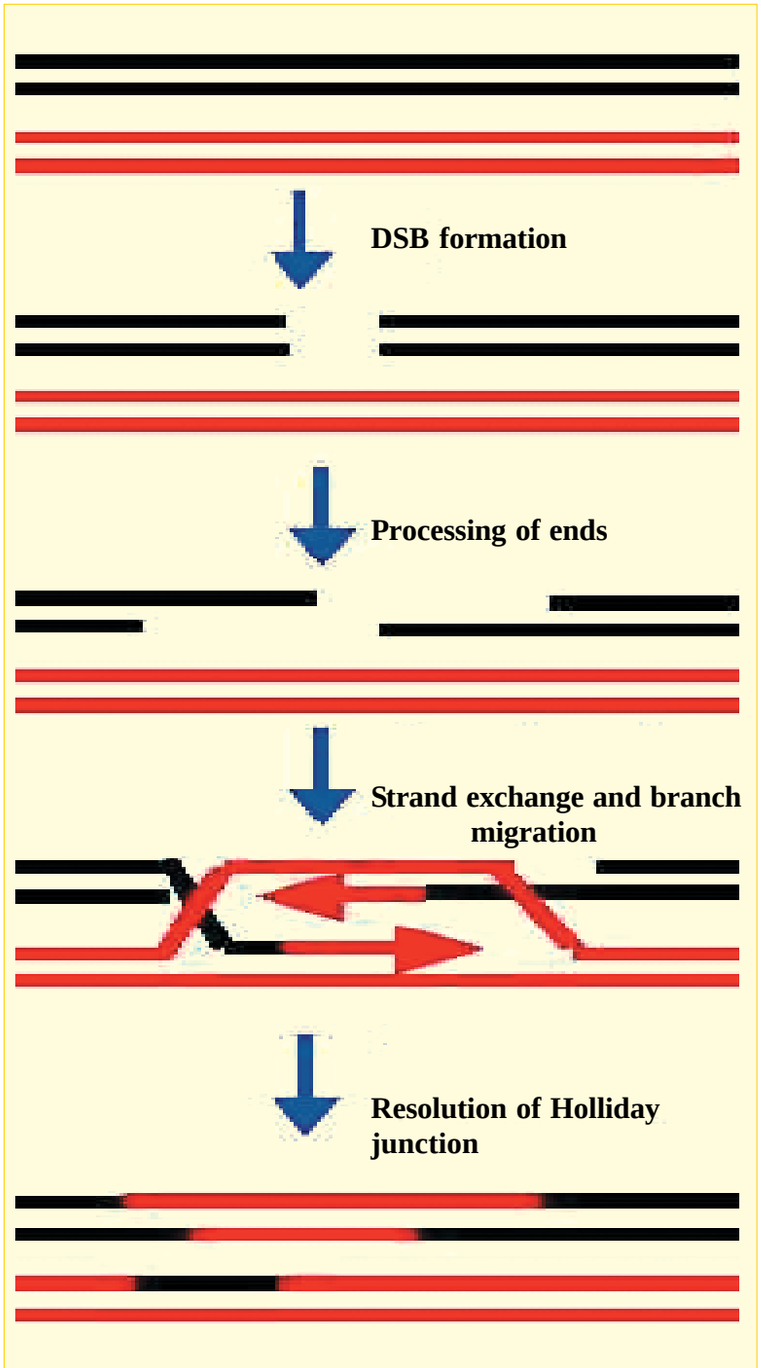
• **Non-homologous end joining (NHEJ) :** Double strand breaks (DSB) in DNA are formed during the exposure to ionising radiation and also during normal physiological processes. These strand breaks are lethal to the genome if not repaired. DSBs are repaired by non homologous end joining. However, during this process the genetic information may be lost. In the first step Ku and DNA dependent protein kinase (DNAPKcs) bind to DNA ends. End alignment takes place due to micro-homology of about 4 nucleotides. Gaps are filled by DNA polymerase and the following ligation reaction, catalyzed by XRCC-4-DNA ligase IV complex.




*Defects in the DNA repair genes are known to be associated with diseases such as in Xeroderma pigmentosum, Wilson's disease, Werner syndrome, Bloom's syndrome, Cockyane syndrome, Ataxia and different cancers.*

essential for repair of DNA lesions following ionizing radiation.

DNA polymerase I is an important enzyme in DNA repair. Structure-function studies identified the domain containing Arg–682 having a major role in template-dependent dNTP binding and polymerization. This was later confirmed using site-directed mutagenesis.




*DNA Double strand break, recombination and repair*

## Enzymology

For several decades the research in biochemistry was dominated by enzymology. Enzymes of different metabolic pathways involved in carbohydrate and nucleic acid metabolisms were purified from several organisms such as *Halobacteria, Corynebacteria , Cyanobaceteria, Lactobacilli, Streptococci* as well as from plant and mammalian systems and their catalytic and regulatory properties were investigated. These studies involved molecular characterization, unraveling mechanism of action, active site mapping and regulation. During the early years, several new enzymes such as L aspartate ammonia ligase (6.3.1.4), indole : oxygen 2,3 oxidoreductase (1.13.1.17), 2-aminophenol : oxygen oxidoreductase (1.10.3.4), anthranilate tetrahydropteridine : oxygen oxidoreductase (1.14.16.3), Catechol : oxygen oxidoreductase ( 1.1.3.14), succinyl-CoA : glycine C- succinyl transferase (2.3.1.37) were discovered and also novel metabolic pathways were revealed. Structure-function relationship was also investigated using the technology of cloning and site-directed mutagenesis in some enzymes. Investigations were also undertaken to immobilize enzymes for biotechnological applications. Some of the interesting findings related to: 1)induction of alkaline phosphatase(a periplamic enzyme) in *E.coli*, 2) reverse and forward reactions of carbamyl phoshokinase from *Streptococcus faecalis* R., 3) extracellular and intracellular nucleases in *Staphylococcus aureaus and* 4) cloning of the gene of an inducible beta-galactosidase of *Corynebacterium murisepticum*.

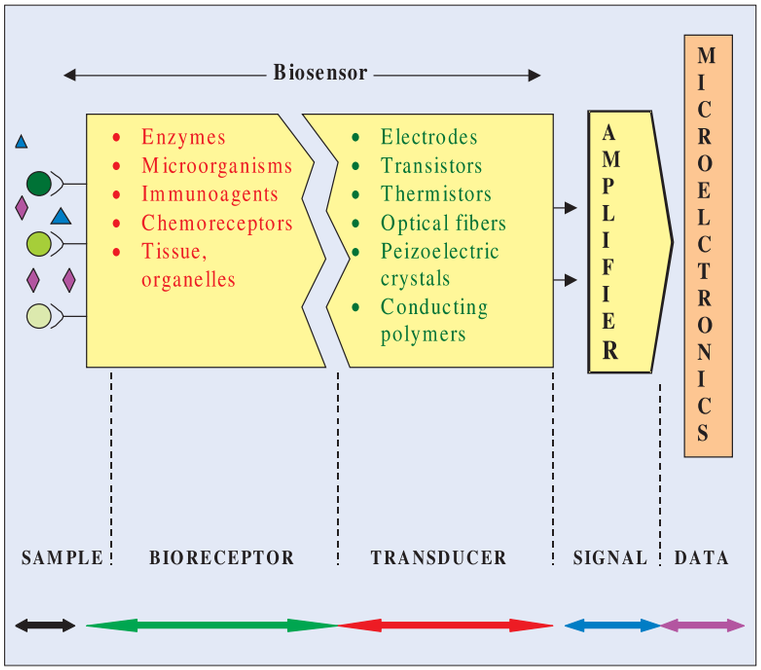
Genes and enzymes involved in utilization of glucose and regulatory control of this energy-yielding pathway in yeast were identified. Brain hexokinase was investigated to elucidate structure-activity relationship and to understand its regulatory functions at the molecular level. Such investigations required inter-disciplinary approaches and nuclear magnetic resonance as well as electron spin resonance spectrscopy were harnessed to elucidate protein structure-function relationship. The active site residues involved in transfer of a phosphoryl group from ATP to glucose were identified and mechanism of regulation of hexokinase by phosphate and glucose 6-phosphate were examined.

## Enzyme Technology

• Enzymes are work horses of living systems. Enzymes carry out all the complex chemical reactions associated with "life" in precise and well-regulated manner at optimum temperature, pressure, pH and osmotic pressure required for growth of the respective organism. Use of enzymes as catalysts in chemical industry will lead to increase in efficiency and precision, besides reducing energy cost and environmental pollution. It may also replace a number of technologies based on chemical syntheses to more ecofriendly, non polluting green chemical routes.

• Techniques were developed in BARC for immobilization of enzymes for continuous use in bioprocessing industry under more stabilized conditions. It was observed that immobilized permeabilised/non-viable cells were an economical source for invertase, catalase, D-amino acid oxidase, urease and alcohol dehydrogenase. A variety of new techniques were developed for immobilization of enzymes as well as cells. Some of these include use of hen egg white as a proteinic support, *Ocimum basilicum* seeds as a pellicular support; immobilization of biocatalysts in radiation polymerised acrylic and gelatine supports; entrapment in natural bioploymer gels, conjugate gels, open pore gels and inorganic cementing materials. Others include, preparation of fungal/yeast biomass mats using highly porous synthetic and natural foam materials; magnetization of biomass; stabilization of biomass through cross-linking and surface immobilization of biomass as biofilms through adhesion using polyethylenimine on synthetic and natural polymers.

• Immobilized enzymes and microbial systems were investigated for preparation of lactose free milk, removal of glucose from eggs prior to spray drying, in the preparation of α-keto acids, gluconic acid and in development of cofactor regenerating systems at lab scale. Extensive studies were carried out for preparation of invert sugar syrups from cane sugar using immobilized yeast cells. Immobilized cells are also being evaluated in bioremediation of radionuclides from liquid wastes.




## Biosensors

- Biosensors combine the selectivity and sensitivity of biology with the processing power of modern microelectronics to offer powerful new analytical tools with major applications in medicine, environmental diagnostics and food industries.

- A biosensor consists of a biological sensing element such as an enzyme, antibody, cell, cellular organelle, chemoreceptor, tissue or biomolecule immobilized in close contact with a transducer such as an ion specific electrode, thermistor, optical fibre, piezoelectric crystal, silicone wafer, conducting polymer etc.

- Biological signal arising due to its interaction with the analyte can result in an electronic or allied signal that can be easily measured, amplified and documented.

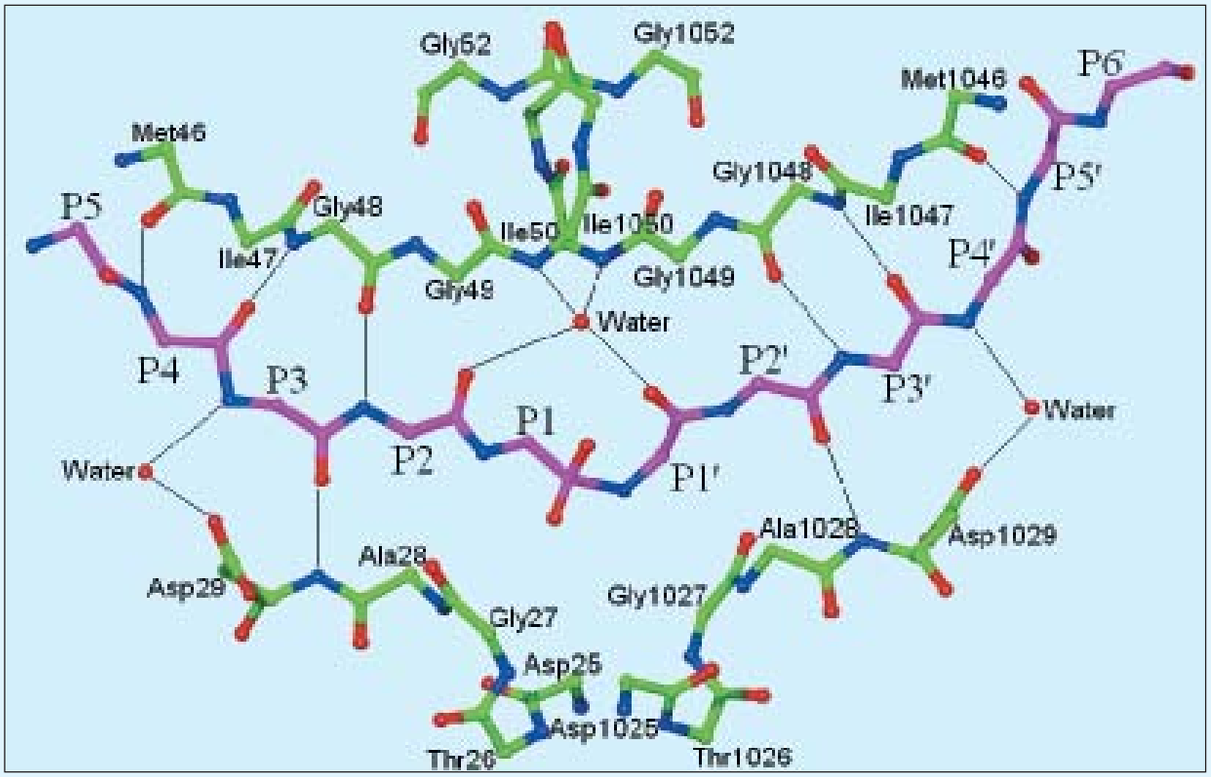
- A porous silicon based novel capacitative immunosensor based on Electrolyte-Insulator-porous Silicon (EIS) is being designed and fabricated in BARC.

# Structural Biology

## Link B9

*Biological function is a complex interplay between a number of different types of biological molecules, especially nucleic acids and proteins. Understanding of the structure, dynamics and interactions of these molecules is crucial for development of curative agents against organ malfunctions and diseases. Structural Biology encompasses highly diverse disciplines such as x-ray crystallography, electron microscopy, computational biology, nuclear magnetic resonance, molecular modeling, protein engineering, designer bio-molecules etc. Knowledge of three-dimensional structures of molecules and their dynamics are basic requirements for understanding their function. Though structural biology suffers from limitations of a reductionist approach, it has led to major path-breaking discoveries in life sciences. The most important among these was determination of the structure of DNA by X-ray diffraction. The two-stranded helical structure of DNA not only immediately explained how a living system could pass on its traits to the progeny, a feature unique to living beings, but it also spawned the disciplines of molecular biology and biotechnology. Single crystal X-ray diffraction studies on proteins can characterize disease conditions at an atomic level, enabling development of effective remedies through the process of structure-based molecular design. Crystal structures of several proteins are now known. The topography of the active sites of several enzymes has been understood. Unraveling of the 3 dimensional structure of major histocompatibility antigens in 1987 was a major landmark since these molecules are involved in self associated recognition of foreign antigens by the T lymphocytes of the immune system. This technology of protein and ligand engineering is very powerful given its wide applicability, without limitations on the source and size of the molecule under study. It is possible to virtually travel through a molecule using software tools to understand its functions. New NMR techniques and methodologies have been developed for rapid determination of solution structures of proteins and nucleic acids. Another as yet not fully understood phenomenon is protein folding. It is a key to the function of the protein. The role of other proteins in this process remains to be clearly elucidated. The problem of protein folding involves complex mathematical modeling. It is, therefore, likely that exploits in structural biology may help to deal with problems as diverse as bioterrorism and cancer.*

*First Electron Microscope in India fabricated at SINP*




*Interactions of HIV-1 protease with the substrate peptide*

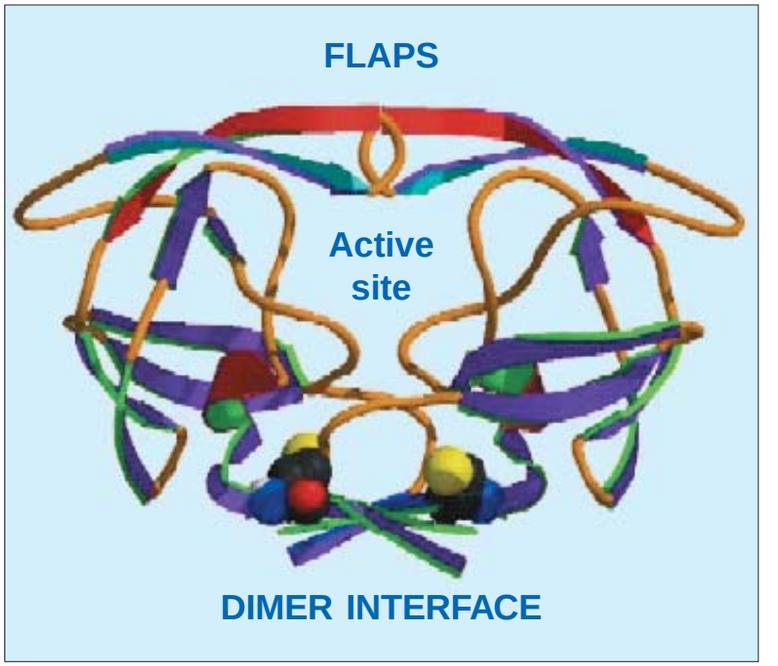
# Structural Biology

Molecular crystallography has been one of the strong areas of research work in BARC and SINP. Recently National Centre for Biological Sciences also has developed programmes on molecular modeling and protein folding. Pioneering studies on structures of amino acids and mononucleotides were carried out at BARC using neutrons produced in CIRUS and DHRUVA reactors. Intensive research was initiated at SINP in X-ray crystallography, aimed at determining the 3-dimensional structure of biologically important molecules in 1959 and in BARC in the year 1978. A major boost to this multidisciplinary research was given with the establishment, in 1996, of the National Facility for Macromolecular Crystallography, in the Solid State Physics Division. Researchers from within BARC and outside have used these instruments to investigate structure-function relationships in a variety of proteins from different sources.

These have now been extended to cover various fields of Structural Biology and Biomolecular Spectroscopy and Macromolecular Crystallography. The complete 3-D structure of a chymotrypsin inhibitor protein was deciphered. Subsequently, high resolution protein structures of more protease inhibitors and other important proteins have been determined. Important contributions were made on the thermodynamics of drug-DNA interactions with special references to aureolic acid group of antitumor antibiotics. Spectroscopic studies on proteins and peptide structure, conformation and folding of enzymes, interaction of chemical denaturants with proteins and lipid-protein interactions in erythrocyte cytoskeletal proteins are being done. Model building studies on the conformation and dynamics of nucleic acids were performed. A microscopic image-based classification scheme for membrane clusters with different lipid compositions has been put forward that could be potentially used in studies on cellular and macromolecular assembly.

Structural studies are being carried out on toxins similar in activity to ricin, proteins from the AIDS virus, and presently on proteins from the malarial parasite *Plasmodium falciparum* at BARC.

Plant toxins such as ricin are protein molecules which can kill cells by inactivating ribosomes. These proteins can and have been used for beneficial and destructive purposes. As components of 'magic bullet' immunotoxins, they can be used to specifically kill tumor cells thereby providing cure for different types of cancers. The effectiveness of the immunotoxins can be enhanced many fold through structural inputs from X-ray crystallography. The structure of gelonin, a plant toxin with activity similar to that of ricin has been determined at 1.8Å resolution.



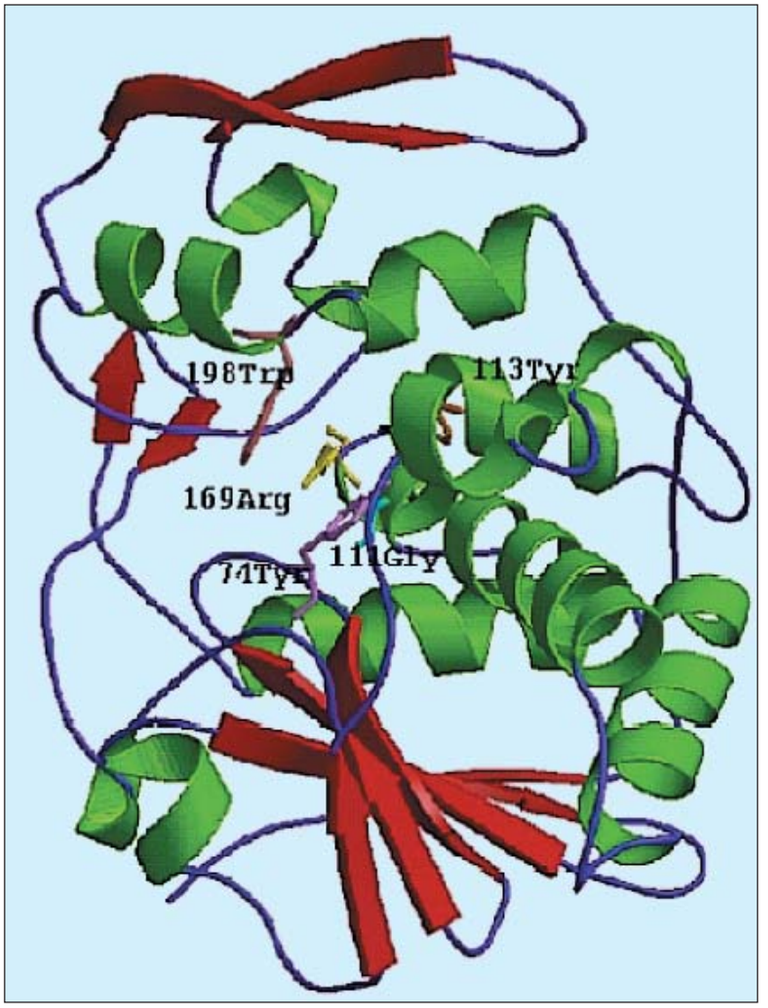

*Ribbon diagram of HIV-1 protease, the target for drug-design against AIDS. Unliganded closed-flap structure was discovered at BARC*

### National facility for macromolecular crystallography

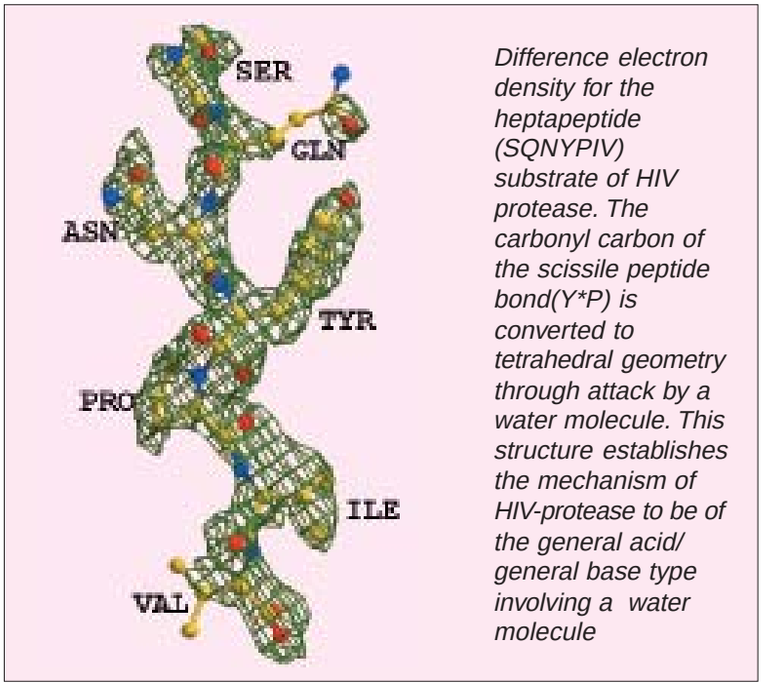
This Facility is equipped with state-of-the-art instruments for

- Sample preparation using molecular biology tools
- X-ray diffraction data collection
- High-speed computation and computer-graphics

Researchers from within BARC and outside have used these instruments to investigate structure-function relationships in a variety of proteins from different sources.




*Ribbon diagram of the RIP, Gelonin solved to 1.8Å resolution at BARC*




*Difference electron density for the heptapeptide (SQNYPIV) substrate of HIV protease. The carbonyl carbon of the scissile peptide bond(Y*P) is converted to tetrahedral geometry through attack by a water molecule. This structure establishes the mechanism of HIV-protease to be of the general acid/ general base type involving a water molecule*

This structure could provide the template for design of detector molecules, through the process of structure-based drug-design, which can detect deadly toxins belonging to the family of ricin. Further, the structure of gelonin can help in design of proper conjugates between gelonin and cell-recognising antibodies, so that specific types of tumor cells can be killed.

Acquired Immune Deficiency Syndrome (AIDS) is projected to wreak unimaginable havoc and therefore, there is an urgent need to develop methods of killing the AIDS virus. The protease enzyme from the virus is a key enzyme essential for viral propagation. This enzyme is a target for inhibitor design world-wide. The work on HIV-1 protease has shown that under specific conditions of crystallization, one can make the flaps of HIV-1 protease adopt closed conformation even in the absence of any ligand bound within the active-site.

This feature of the molecule can then be exploited for rapid development of inhibitors by the crystallographic method. In addition, this discovery has allowed the study, for the first-time in the world, of structures of the protease complexed with two oligopeptide substrates. In both these structures, the substrate has been converted into a tetrahedral reaction intermediate through nucleophilic attack by a water molecule. This novel result provides crystallographic evidence that helps to decide the correct mechanism of action of HIV-1 protease. The interactions between the protease and the reaction intermediates provide a breakthrough in the design of very potent inhibitors of this enzyme. Such inhibitors could then be developed as drugs against AIDS.

## Electron microscopy

Under the inspiration and support of Prof. Meghnad Saha and Prof. N. N. Dasgupta an electron microscope, the first of its kind in India, was fabricated indigenously in the Biophysics Division of Saha Institute of Nuclear Physics. Pioneering work was done on electron microscopic characterization of infectious microorganisms, different cellular architectures, proteins and nucleic acids.The technique of ultrathin sectioning was developed in early 1950 that made possible the identification and characterization of *Escherichia coli*, *Mycobacterium leprae*, malarial parasites, microbes responsible for Kala azar (*Leishmania donavani*) and several other protozoa at the ultrastructural level. Structural organizations of nucleoproteins,

ribosomes and coliphage T7, human hemoglobins A, F and E, serum albumins and *E. coli* DNA were analyzed.

### At the interface of physics, chemistry and biology

Current research trends in physical sciences are looking up to biology to understand how biological systems are built so precisely molecule by molecule, and how these molecules self assemble in protein machines.

Scientists at TIFR are unraveling the principles of biomolecular conformation, developing of methodologies for elucidating three dimensional structures and dynamics of molecules in solutions, understanding the roles of metal ions in the functions of different enzymes and other cellular functions, unraveling the metabolic pathways in living cells, developing new biophysical techniques for visualizing functions inside brain cells, and probing chemical reactions and molecular associations at the most fundamental level. New NMR techniques and methodologies have been developed for rapid determination of solution structures of proteins and nucleic acids. With novel algorithms (TANDY, CHORDS, TATAPRO) a number of structures of biological macromolecules have been determined in aqueous solutions to atomic resolution, and these have provided great insights into biomolecular recognition and function. An important question is: how does a protein fold to its native structure within a short time of a few milliseconds? The conformational space of a protein has millions and millions of conformations. How protein chooses a right conformation has been an intriguing question in biology. Scientists in TIFR and NCBS have made important contributions in these developments and identified folding transitions in different proteins.

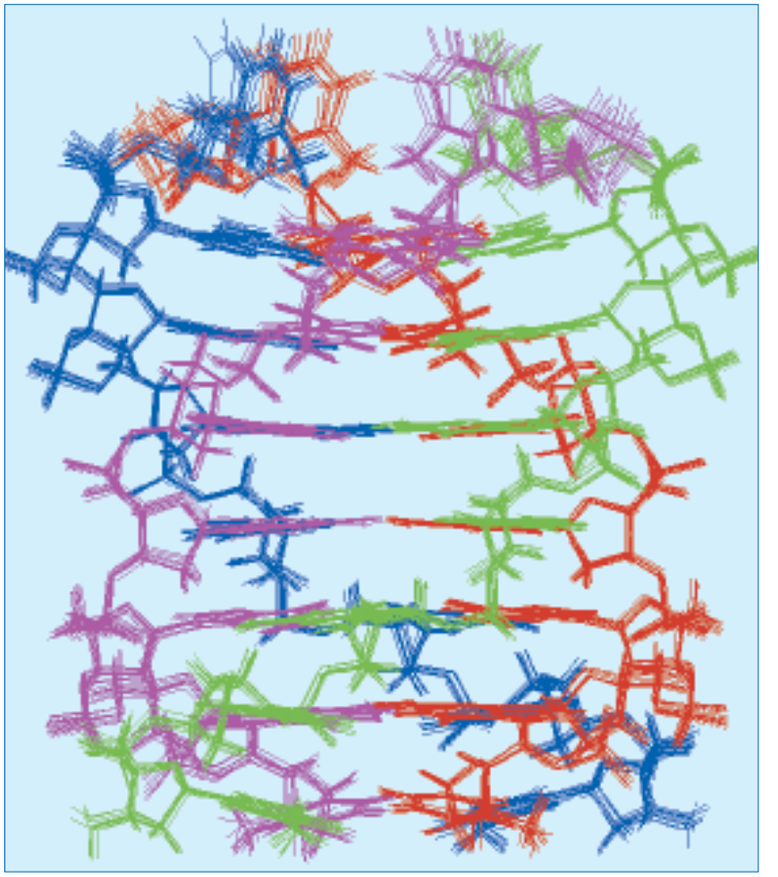
Spectroscopic techniques such as electron paramagnetic resonance, relaxation and ultrafast laser techniques are used for studying molecular basis of biological systems. The objective of this research is two-fold: to understand the correlation between molecular structure and spectroscopic and luminescence properties, and to use molecules as fluorescent probes to understand the properties of the medium that surrounds the probe. Binding of the antimalarial drug, quinine to heme was

*Ribbon drawing of the structure of recombinant human carbonic anhydrase solved in BARC*

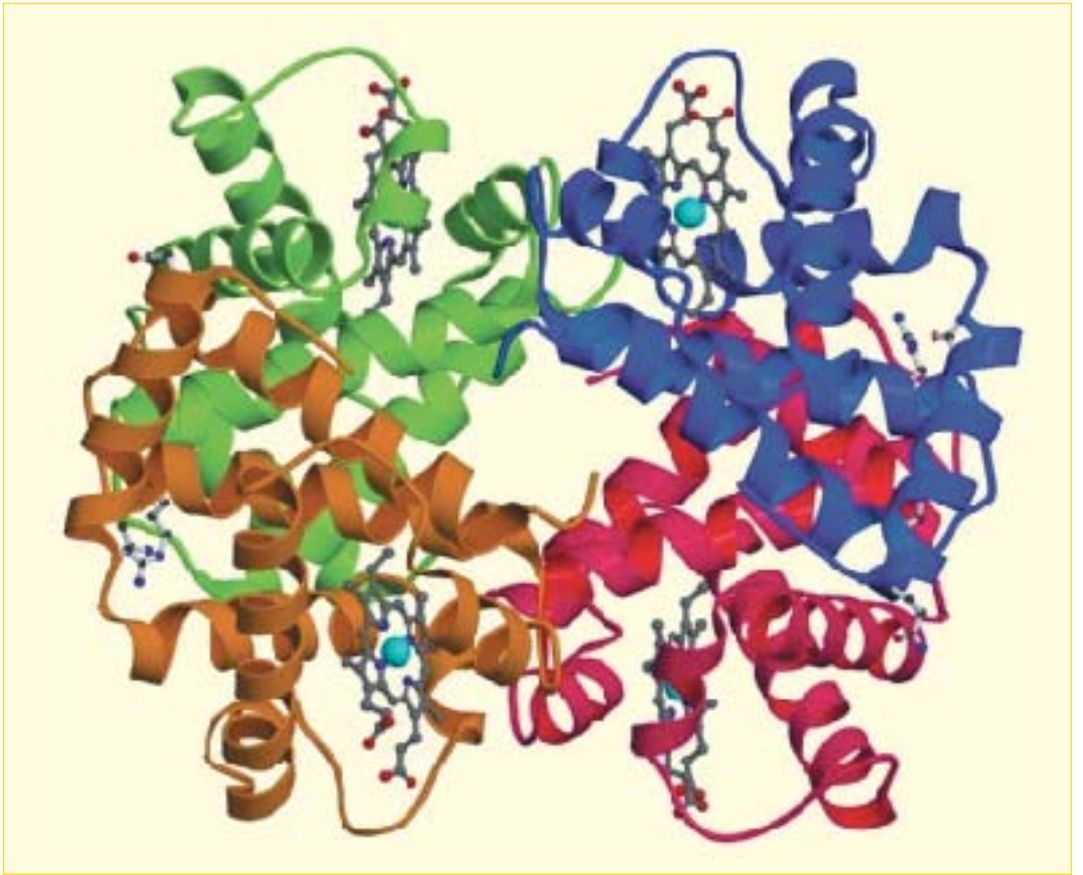
*Ribbon drawing of the structure of Lysozyme refined in BARC*

determined by NMR techniques, which confirmed that the heme released on degradation of hemoglobin indeed acts as a receptor of the antimalarial drug.

Several vital biochemical processes such as drug metabolism, hormone biosynthesis and detoxification of many xenobiotic substances are carried out by a class of iron containing enzymes known as cytochrome P450. These enzymes show high substrate specificity and product selectivity. Scientists in the TIFR have identified distinct conformational changes in the enzyme during binding of the substrate and helped to determine the substrate access channel of the enzyme. Two distinct stable conformational forms of the enzyme could be stabilized in solution, which are responsible for slip in the catalytic cycle of this vital enzyme.



*The quadruplex structure of a DNA sequence belonging to the telomeres, which play important roles in cancer and ageing, determined at TIFR*



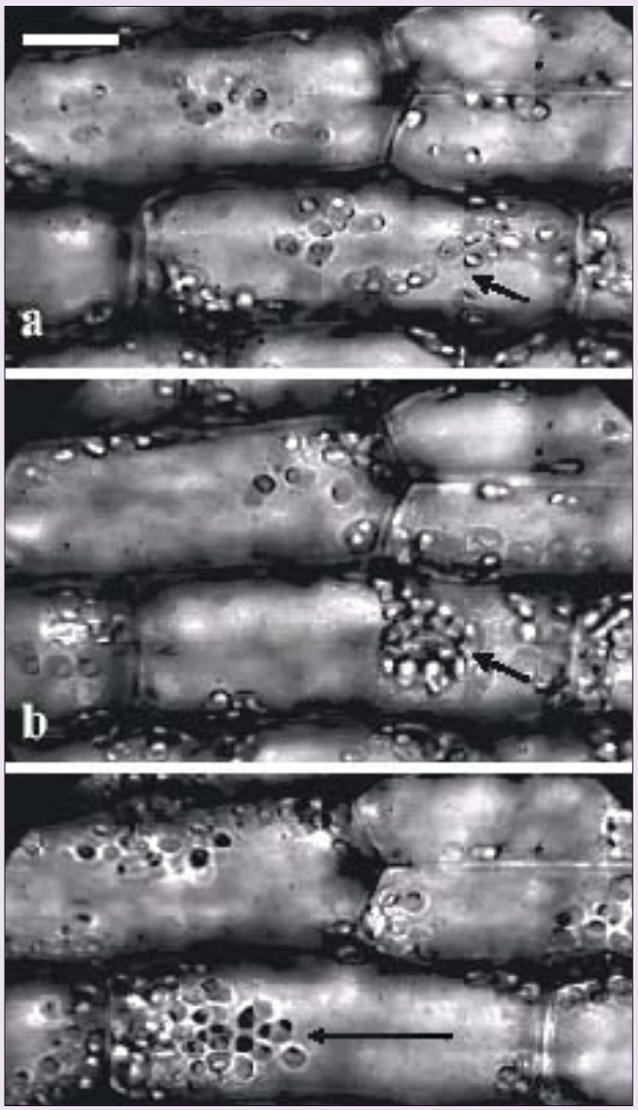
*3D structure of hemoglobin A2, implicated in beta thalassemia, at 2.1 A resolution, determined at SINP*

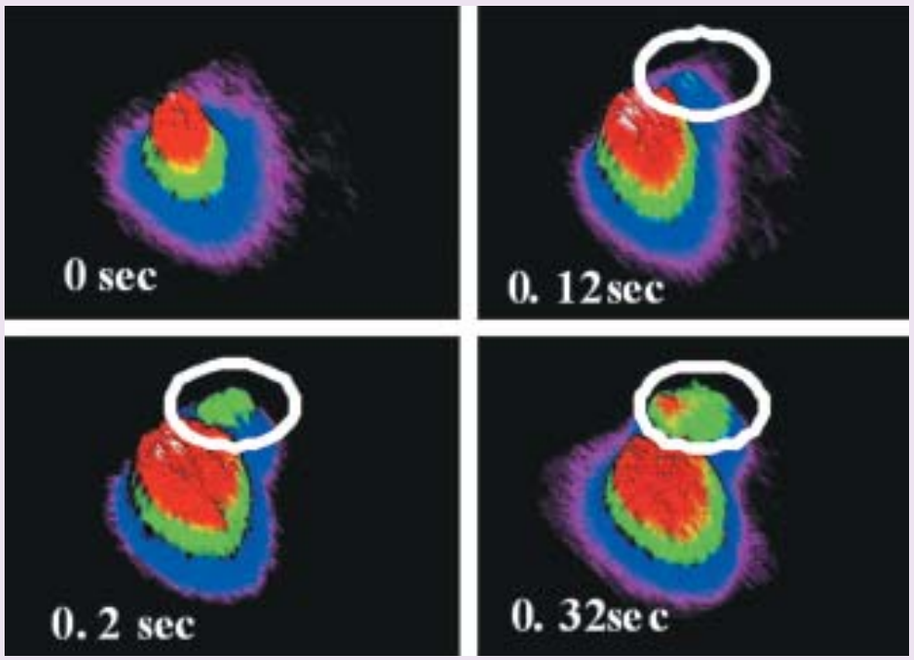
# Lasers in Biology

## Link B10

*Light has been used for therapy since ancient times. Some well-known examples of the therapeutic use of light are in the treatment of psoriasis, neonatal jaundice, skin tuberculosis, photodynamic therapy of cancer, etc. More recently, several researchers have reported that irradiation with an intense narrow bandwidth light (laser) can have profound effects on cellular cultures and animal models and can also lead to a variety of therapeutic effects in humans like accelerated wound healing, treatment of pain of different origins, etc. Although the usage of lasers for non-surgical therapeutic applications is growing it has not yet become an established clinical modality because mechanisms for many of the photo-therapeutic effects are not very well understood. The clinical potential of this rather simple and inexpensive therapeutic modality is motivating considerable work in this direction and substantial progress is being made. For optical diagnosis one mostly makes use of the light scattered from a tissue. Most of the incident light is scattered without any change in frequency. Even so, a very weak component is scattered inelastically i.e. with a change in frequency via processes like fluorescence, Raman scattering etc. This inelastically scattered light is characteristic of the chemical composition and morphology of the tissue and may, therefore, help monitor onset and progression of diseases like cancer that are often associated with biochemical and morphological changes in the tissue. However, since the inelastically scattered light is a very small fraction of the incident light, practical applications based on this component require use of high spectral brightness source like lasers and appropriate light delivery and collection systems. Laser spectroscopic techniques hold considerable promise. Further, these techniques have the potential for in situ, near real time diagnosis and the use of non-ionizing radiation ensures that the diagnosis can be made repeatedly without any adverse side effects. Other promising areas of application of lasers in biology are for manipulation of cellular organelles, motor-cargo interaction in neurons and studies on protein aggregation in neurological diseases.*

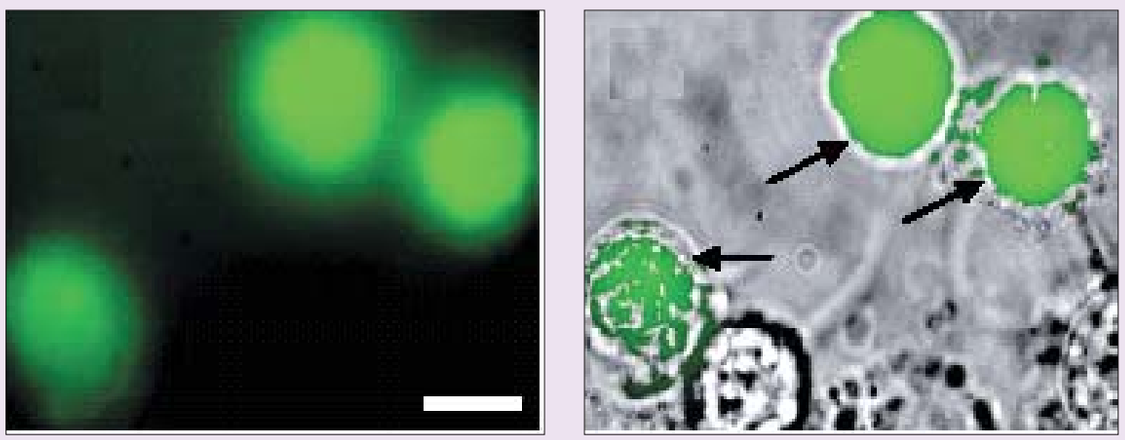
# Intracellular optical trapping and 3D orientation using lasers




*Intracellular 3D orientation and auto fluorescence imaging of motile dividing chloroplasts*

*Intracellular optical trapping, isolation and displacement of chloroplasts in Elodea densa with a CW 1064 nm NIR laser. Time lapse video images showing the position of chloroplasts in dark-adapted living leaf cells (a) and at different times (b and c) after switching on the NIR trapping beam focused at a particular locus. (bar= 20* μM*)*



*Fluorescence image of MCF-7 cells 24h after transfection with green fluorescent protein (GFP) coding plasmid (bar= 10* μM*)*

# Lasers in Biology

Promotion of medical applications of lasers is one of the objectives of the Centre for Advanced Technology (CAT) in Indore. Effects of laser radiation, or narrow bandwidth light from other sources, on cellular cultures and animal models, with and without exogenous photosensitizers are being studied. Lasers are also used for manipulation of microscopic objects, as optical tweezers for microfluidics applications, for microinjection of molecules in cells and for optical imaging through turbid medium. At TIFR another group has initiated studies combining lasers and microscopic techniques to address some basic mechanistic questions in neurobiology.

## Effect of laser irradiation on cell cultures and animals

Laser beam irradiation is beneficial to patients of pulmonary tuberculosis as it helps to heal cavities. Nitrogen laser irradiation is being actively used at Choithram Hospital Research Centre, Indore for faster healing of wounds. Mechanisms responsible for these therapeutic effects are not well understood. Studies on the effect of $N_2$ laser irradiation on the skin of albino rabbits and mice showed that at certain doses it can lead to proliferation of cells in the active epidermal layer. This may contribute to faster healing of wounds. However, higher doses caused inhibition of cell proliferation. It was important, therefore, that parametric dependence of this phenomenon was carefully evaluated in order to elicit the desired clinical response. $N_2$ laser irradiation of red blood cell lysate induced oxidation of hemoproteins. Experiments with deoxygenated red blood cell lysate rule out involvement of any reactive oxygen species. This suggested that the process is photochemical but not photodynamic. Further, since macrophages play an important role in wound healing, effect of light on macrophages was also studied. He–Ne laser irradiation led to stimulation of macrophage activity. Single cell gel electrophoresis (Comet Assay) of He-Ne laser irradiated B lymphoblast cells demonstrated a reduction in UV A induced DNA damage. Similar observations were made in optically trapped cells and in mouse peritoneal macrophages.

When these studies were extended to the bacterial species, *E. coli,* it was seen that respiratory electron transfer process was stimulated by He-Ne laser irradiation through change in redox state of respiratory components. Secondly, He-Ne laser pre-irradiation induced protection against UV-C irradiation in several *E. coli* strains.

Antibiotic resistance of bacteria is a growing concern. Photodynamic inactivation of an antibiotic resistant strain of *Pseudomonas aeruginosa* was achieved by use of endogenously synthesized porphyrins induced by amino levulinic acid (ALA). These findings may be useful for inactivation of antibiotic resistant strain of *Pseudomonas aeruginosa* infecting burns, wounds and bacterial lesions in patients. He-Ne laser irradiation of *Bacillus subtilis* in presence of methylene blue (MB) and toludine blue (TB) showed photodynamic generation of singlet oxygen leading to decrease in cell membrane fluidity.

## Optical imaging through turbid medium

Optical techniques provide sub-millimeter resolution imaging without the need for ionizing radiation. However, in contrast to x-rays, optical photons are strongly scattered in tissue leading to a blurring of the image. Several approaches can be used to pick out the useful image bearing light from the background of multiply scattered light. These exploit depolarization or loss of coherence of the scattered light. Further, scattered light emerges from the tissue in all directions. It also takes longer time to emerge as compared to the unscattered (ballistic) or predominantly forward scattered (snake like) components. The latter essentially travels in forward direction and so arrive earlier. Coherence gating filters out ballistic photons having the highest image information and hence can provide images with the best resolution (down to a few mm, limited by the coherence length of the source). However, the number of ballistic photons decrease very rapidly on propagation through a turbid medium and are of the order of $e^{-100}$ of the incident photons on propagation through 1cm thick tissue with a scattering coefficient of ~$100cm^{-1}$. Therefore, coherence gating can only be used for high resolution imaging of transparent objects (like ocular structure) or thin turbid tissue like mucosal layers of hollow tubes. Multiply scattered light can also be filtered out using spatial filters and by use of ultra-short temporal gates (of picosecond / sub-picosecond duration). In this approach both ballistic and forward scattered components are filtered out. The use of both ballistic and snake like components facilitates imaging through larger

## Laser micromanipulation of microscopic biological objects

Use of lasers for precise manipulation and processing of microscopic objects, such as a single living cell or even objects within a single cell, is finding widespread applications in biological research and technology. The two important tools for laser-assisted micromanipulation of microscopic objects are laser microbeam and laser optical trap. Laser microbeam is essentially a pulsed laser beam coupled to a microscope. The large intensities (~ $10^{13}$W/cm$^2$) generated at the focal point of the large numerical aperture microscope objective can be used to cut, perforate or fuse microscopic objects with sub-micrometer accuracy. As these intensities arise only at the focal point, it is possible to work within the depth of a transparent object without opening it. Optical tweezers or laser optical trap uses the light of a continuous wave infrared laser for precise manipulation of microscopic objects. Here, the gradient forces arising due to the large gradient of light intensity in the focused laser beam are used to trap microscopic objects at the focal point of the laser beam. Unlike mechanical microtools, the optical trap is gentle and absolutely sterile and can be used to capture, move and position single cells or sub cellular particles without direct contact or significant damage.

A laser micromanipulation set up has been developed at CAT in which several novel micromanipulation techniques have been incorporated including controlled 2D and 3D rotation of trapped objects and for acceleration and projection of trapped objects. The laser micromanipulation set up has been fully characterized and has already been used for a variety of studies ranging from malaria diagnosis, laser assisted enhancement of neuronal growth cones, 3-D viewing of dividing chloroplasts, microinjection of impermeable dyes into cells, transfection and microfluidic applications.

depths of the turbid medium. Further, with the use of non-linear optical time gating techniques like stimulated Raman scattering, the gated light can be amplified to generate reasonable signal levels even after propagation through few cm thick tissue. The resolution, however, is poorer (of the order of 100 μm). Another approach, referred to as inverse source approach for optical imaging is based on detection of multiply scattered light at various positions around the object. From the measured transmitted intensities and known optical properties, one can, in principle, generate a spatial map of the absorption and scattering coefficients leading to imaging of any object hidden inside the tumour. Though the spatial resolution possible is rather limited (at best a few mm), there is a considerable interest in this approach because it allows imaging through the largest depths of the turbid medium. Work is being carried out on several complementary approaches for optical imaging through turbid media using time gated optical imaging with Stimulated Raman Scattering (SRS), free space optical coherence tomography to create topographic and tomographic images of non-scattering samples with resolution of <10 μm and polarization gated optical imaging.

Developments related to laser micromanipulation techniques such as Rotating line optical tweezers (RLOT) generated by placing a rotating cylindrical lens in the path of the trapping beam, special techniques for laser assisted 3D rotation of microscopic objects and transportation and acceleration of particles with laser beam are being currently pursued.

### Effect of laser micromanipulation on biological objects

Even with lasers operating in the near infrared spectral range (800nm–1100nm) where cellular constituents do not have significant absorption, the possibility of adverse effects on the cells being manipulated is a matter of concern and requires careful monitoring. With a view to find wavelengths which would lead to minimal damage, studies were carried out on single strand DNA breaks in optically trapped cells. Over the wavelength range of 750 – 1064nm, the threshold for single strand breaks in DNA was observed to be lowest at around 760nm. For irradiation conditions investigated (60-240mW, 10-50J/m$^2$; 30-120s irradiation) the fraction of damaged DNA was a factor of two larger in the wavelength range of 750-780nm as compared to that in the wavelength range of 800-1064nm.

### *In vivo* trapping and 3D sorting of intracellular organelles

Optical tweezers were used for *in vivo* trapping and 3D sorting of organelles in single cells within intact plants of an aquatic macrophyte *Elodea densa*. A single chloroplast could

be optically trapped and displaced in the cell without affecting the other streaming chloroplasts. This implied absence of any interconnection between chloroplasts. However, when the trapping beam with a power of ~ 80 mW was parked at particular loci within the cells for 2 to 3 min, it caused convergence of motile chloroplasts into the trap when they passed through the vicinity. All the chloroplasts so collected in the trap could be moved to one end of the cuboidal cell. Furthermore, relatively smaller organelles, namely proplastids, could be three dimensionally transported elsewhere above the imaging plane. Micromanipulation of functional chloroplasts and proplastids within the target cells could be performed over an extended period of time of up to 30 min without compromising viability of the target cells. Demonstration of intracellular optical micromanipulation of different organelles in intact living plants can have potential applications in cell and developmental biology particularly in studies addressing issues of organelle inheritance as well as in gravitropism of roots and other plant organs.

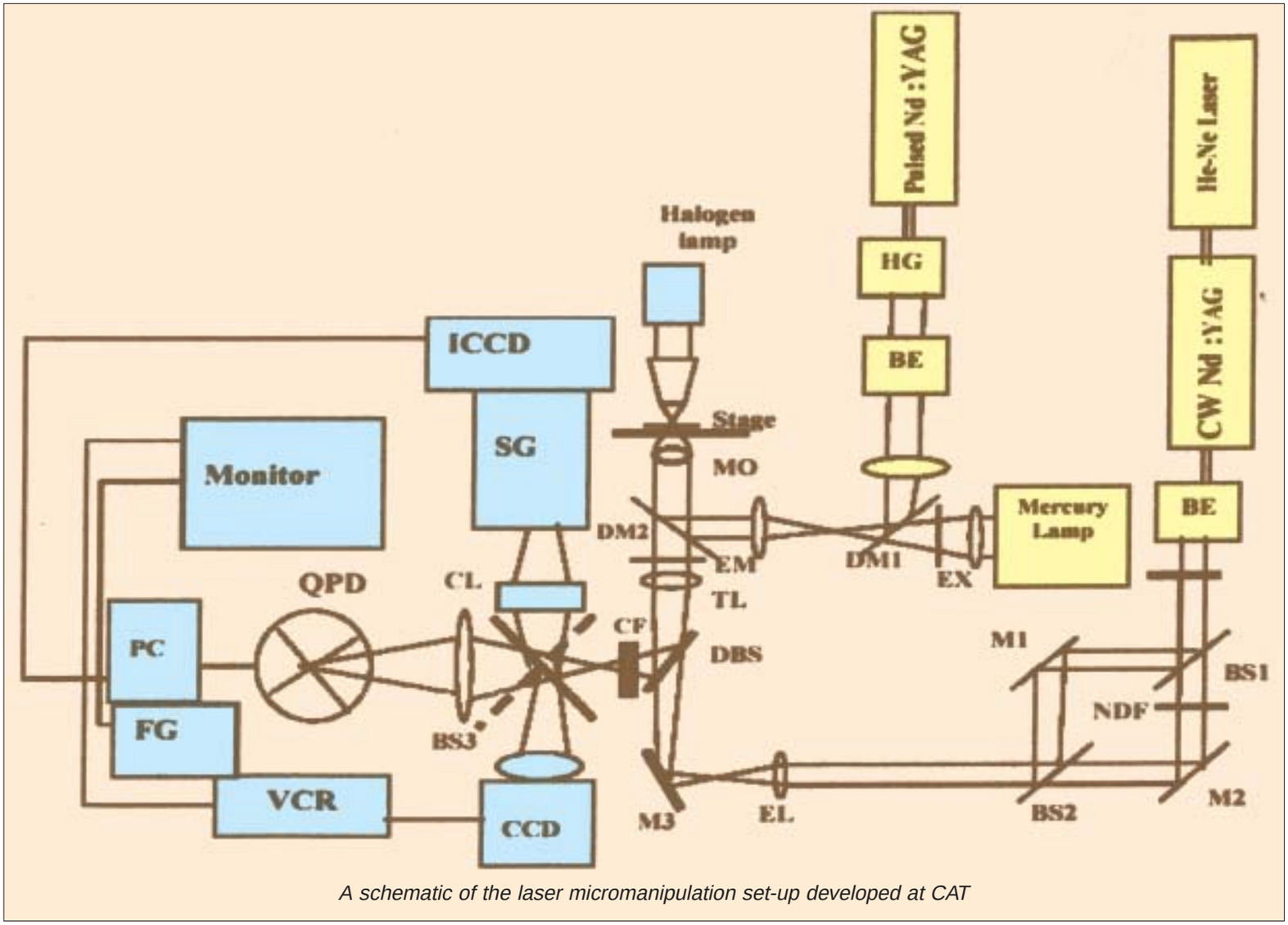
### Use of optical tweezers for microfluidics applications and diagnosis of malaria

The shape of normal red blood cells (RBC) depends on osmolarity of the medium in which it is suspended. In isotonic buffer (~ 290 mOsm) it is bi-concave but in a hypotonic buffer, having an osmolarity of ~150 mOsm, it gets swollen and becomes spherical. On the other hand, in hypertonic buffer RBC takes a meniscus shape. It was found that the meniscus shaped RBC acted as a natural motor when optically trapped. Rotation of these cells occurred because transfer of linear momentum from the trapping beam generated a torque on the cell. The rotational speed was found to increase linearly at lower trap beam powers and more rapidly at higher powers. Deformation of the RBC caused by radiation pressure of the trap beam was seen to be responsible for this phenomenon. Since RBC can be easily transported through microfluidic channels, optical manipulation of RBC can provide a convenient means to perform microfluidics functions. RBC was used as micromotor to pump a liquid with flow rates of few nl/sec to few ml/sec. The direction of flow was determined by the direction of rotation of the RBC motor and the flow rate by the speed of the motor, which could be controlled by varying the power of the trapping beam.

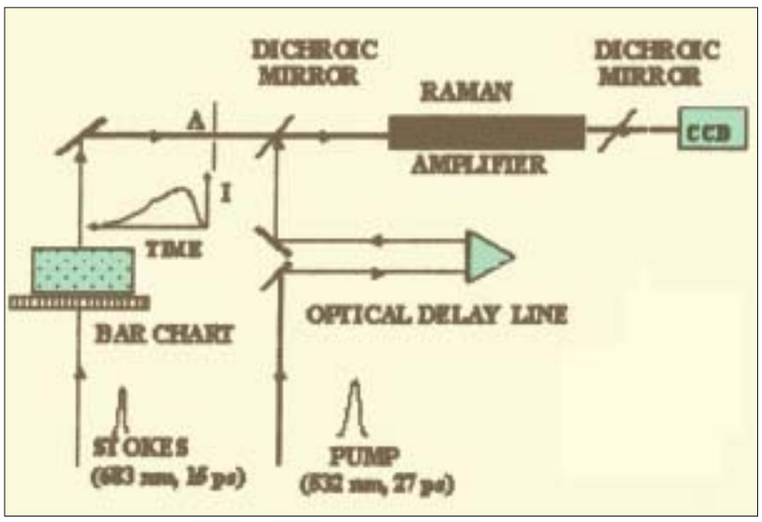
Under the same trapping conditions where normal RBC rotated, RBC having malaria parasite (as confirmed by fluorescence staining) did not rotate. Further, the rotational speed of other RBCs from malaria infected sample, was an order of magnitude lower and also increased much more slowly with increase in trap beam power than that for normal RBC. This difference could be exploited for diagnosis of malaria. Further, it is known that mature parasites release exo-antigens that increase the rigidity of uninfected RBCs in the sample. An important advantage of the present approach is that even those cells from malaria parasite infected blood sample, which do not show acridine orange fluorescence, show large difference in rotational speed as compared to normal cells.

### Laser assisted microinjection into targeted cells

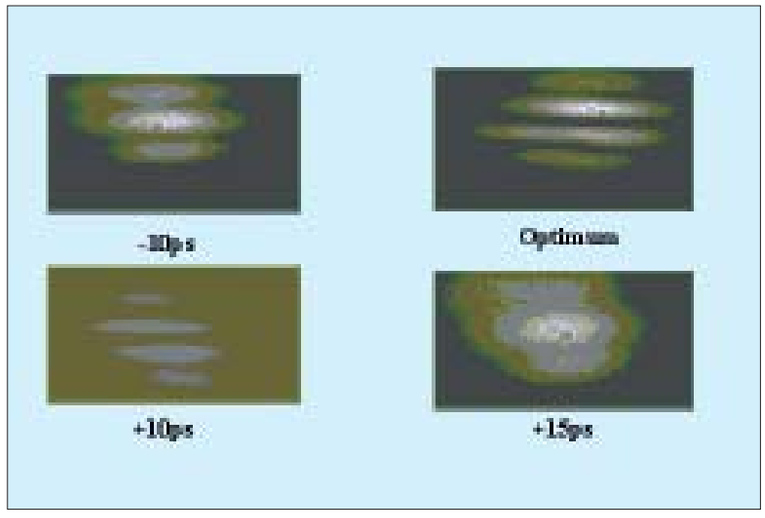
Laser-assisted microinjection (optoporation) of exogenous material (genes, fluorochromes, or photoactivable compounds) into cells is receiving considerable attention. Compared to conventional techniques, optoporation is more efficient and less tedious. It can also be used on cells in suspension as well as attached cells. Constituents of the cell membrane have strong absorption in the UV spectral range. UV lasers were, therefore, the first to be used for optoporation. However, UV can also damage cells or even the transferred exogenous biological material. Use of lasers with wavelength in near infrared region would perhaps be more desirable as cellular components do not absorb them to any significant extent. A nanosecond 1064 nm Nd:YAG laser was, therefore,used for microinjection of impermeable fluorochromes as well as transfection of GFP encoding plasmid into Human breast adenocarcinoma (MCF-7) cells. The Nd:YAG laser beam (17ns, 10Hz) was focused using a 100X Plan Neofluor oil immersion objective at the edge of the membrane of a targeted cell which was suspended in a medium containing impermeable dye merocyanine 540. Optoporation of propidium iodide, another impermeable dye, into MCF-7 cells could also be carried out. Transfection of GFP-plasmid into the cell using nanosecond pulsed laser was also confirmed by monitoring the fluorescence of GFP expressed in transfected cells.




*A schematic of the laser micromanipulation set-up developed at CAT*




*Experimental set-up for time gated imaging using Stimulated Raman Scattering*



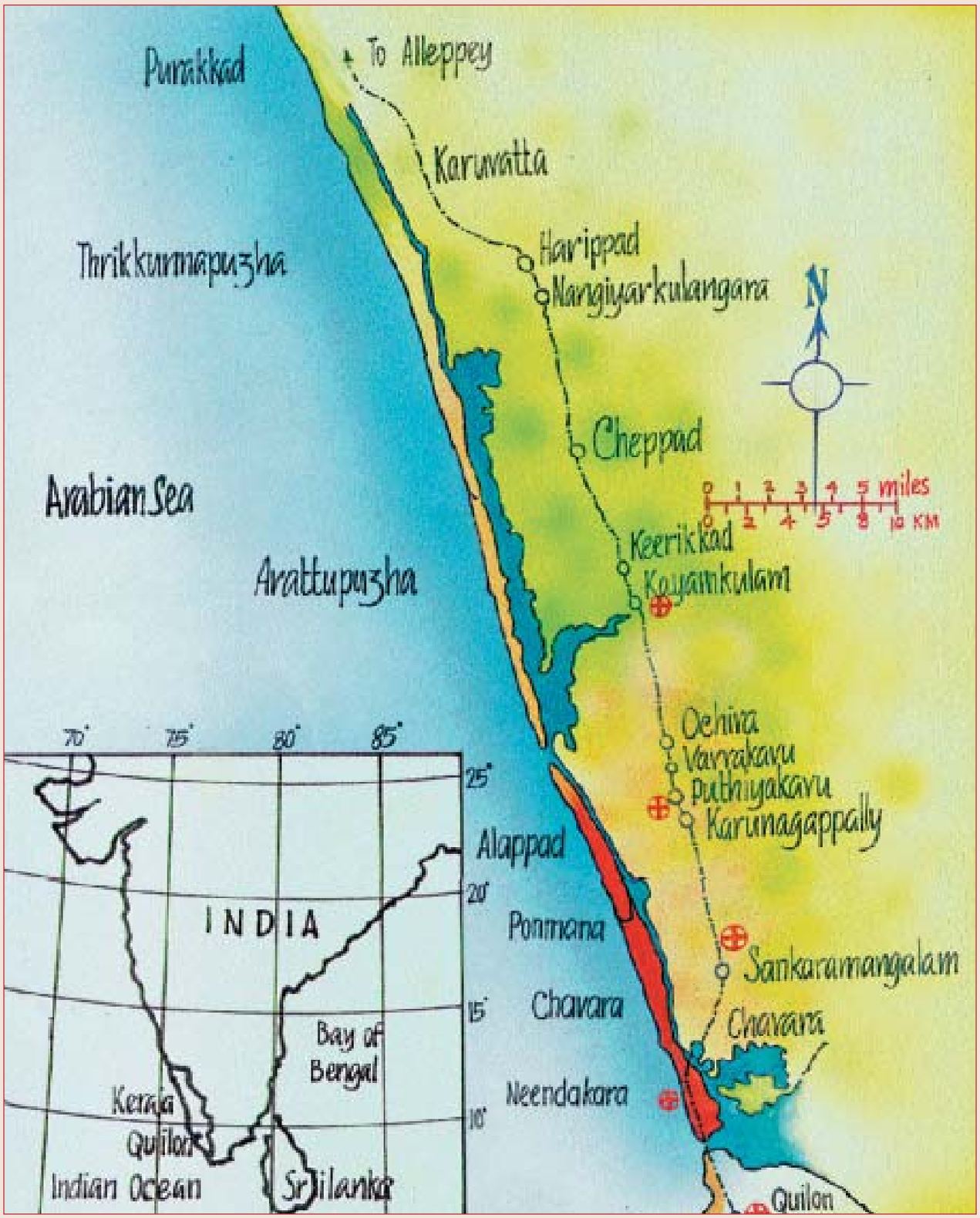

*Images of a bar chart kept in a turbid medium at different optical delays*

# Life in High Background Radiation Areas

## Link B11

*Radioactivity is ubiquitous and omnipresent. Natural radioactivity is present in the soil we live on, foods we eat, water we drink and even the air we breathe. As if this is not enough, we are also exposed to cosmic rays from outer space. Cosmic radiation is a mixture of protons, electrons, alpha particles, and some exotic high-energy particles. Normally, the average annual dose to the world population from all sources is 2.872 mSv out of which 2.4 mSv comes from natural background and only 0.4 mSv from man made sources including medical exposures. This is considered as Normal Level Natural Radiation (NLNR) and the dose varies from <1 to 10 mSv per year depending on the location.The dose received due to nuclear power related programmes is as low as 0.0002 mSv whereas 0.007 mSv is due to atmospheric nuclear testing. People living at very high altitudes receive about 5 times higher dose compared to those living at the sea level. Even frequent air travellers receive an annual dose of 0.01mSv. At some places, nonetheless, particularly those with radioactive soils or rocks, the background radiation levels are many times higher than average. Such high level natural radiation areas (HLNRA) have been identified in different parts of the world. These include Pocos de Caldas, Morro de Forro and Guarapari in Brazil, Yangjiang Province in China, Ramsar in Iran, Chavara- Neendakara in Kerala and Manavalakuricchi in Tamilnadu. These areas are nature's laboratories. People who have lived here for several generations and several hundred years naturally received higher than normal doses of radiation. These regions thus offer a unique opportunity to study low level chronic radiation effects on all stages of human life. Studies on flora and fauna in HLNRA can be useful in the assessment of risk to people and environment in the vicinity of nuclear fuel cycle related industrial activities.*

# High Level Natural Radiation (HLNR) Area in India




*Approx. 30 sq. km strip along the south-west coast of India has interrupted deposits of Monazite sand. (Radiation source - Thorium and its decay products). Radiation dose rate varies widely from <1 to over 35 m Gy per year, (average 4.5 m Gy) in this area*

# Life in High background radiation areas

The monazite bearing coastal belt of Kerala and Tamil Nadu in South West India is a unique HLNR area in many aspects. The population size is approximately 4 lakhs, compared to 73,000 in Brazil, ~1 lakh in China and only 2000 in Ramsar, Iran. Human population in Kerala, as well as other fauna and flora, have survived these chronic exposures to high background radiation for several generations. The level of radiation exposure in HLNR of Kerala varies from <1mGy/yr to 35 mGy/yr, whereas in China and Brazil the radiation levels are almost uniform. In Ramsar, Iran the level of exposure varies from 10 mGy to 260 mGy/yr. The radiation source in Iran is $^{228}$Ra (hot springs), whereas thorium deposits in the monazite bearing sand is the source of radiation in China, Brazil and Kerala in India.

In the late fifties attention of the World Health Organization (WHO) was first attracted towards the possible health and biological hazards of high natural background radiation. Kerala was specially indicated as ideal for such a study. Under Dr. Gopala-Ayengar's leadership a Monazite Survey Project was initiated. Demographic survey and dosimetric studies were launched in the late sixties. BARC has been conducting studies in high background regions of Kerala for over thirty years. A low level radiation research laboratory was established for this purpose at Kollam (Quilon). In the early nineties, the Regional Cancer Centre in Thiruvananthpuram (Trivandrum) set up India's second rural cancer registry in Karunagapally (in Kollam District) to assess the incidence of various cancers in this area and the impact of background radiation on it, if any.

The earliest study, initiated by Dr. Gopal-Ayengar, involved determination of the radiation dose received by the resident population. The dose to which households were exposed was measured. That received by individuals living therein was also determined. Nearly 18 % of the households existing at that time were included in this dosimetric survey and the average external radiation level was found to be around 4 mGy/yr.

A large number of plant species growing in the monazite belt were also included in another survey to assess a) the extent of uptake of radionuclides from the soil by the plants and b) to correlate the magnitude of meiotic abnormalities and pollen sterility observed in these plants with the external radiation level as also the radionuclide content of the plant and

**Some of the high level natural radiation areas in the world**

| Country<br>Location<br>Source | Minimum -<br>Maximum dose<br>(mGy/yr) | Population |
|---|---|---|
| India<br>Kerala<br>Monazite | <1 -35.0 | ~ 400,000 |
| China<br>Yangjiang<br>Monazite | 3.5 - 5.4 | ~ 100,000 |
| Brazil<br>Guarapari<br>Monazite | 5.5 - 35.0 | ~ 73,000 |
| Iran<br>Ramsar<br>$^{226}$Ra | 10.2 - 260.0 | ~ 2,000 |

- Several hot springs also have been found to contain higher levels of natural radioactivity and some of these e.g. Badgastein in Austria and Tuwa in India are used as spas.
- Other sources of enhanced natural radiation exposures in some areas of the world e.g. Sweden, Canada and USA are on account of high levels of indoor radon and its decay products.
- These areas provide an opportunity to study impact of chronic low-level radiation exposures directly on human population through all stages of human development.
- These can, therefore, be considered as

**"Nature's laboratories".**

## Photographs showing normal folk life and high background radiation in Kerala


animal species. In 1966, a collaborative study between Medical Research Council of UK and BARC evaluated dental and skeletal patterns of black rats *Rattus rattus* from this area. A total of 21 parameters, 6 dental and 15 skeletal, were measured on each of the 450 rats trapped from the monazite belt. The rats in the study area received radiation doses 7.5 times greater than those in the control areas. No statistically significant differences between the study and control rats were found.

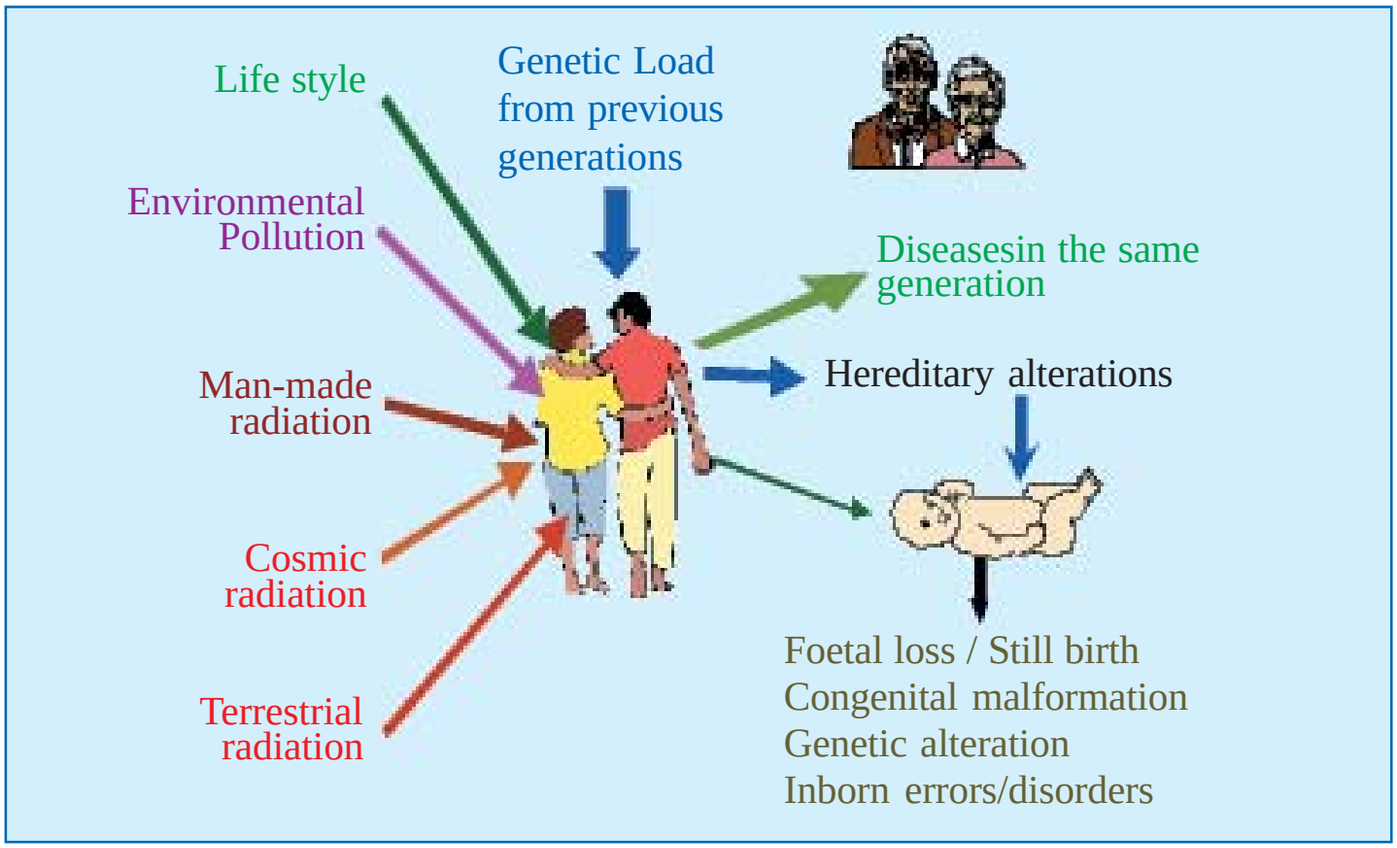
An ad hoc demographic survey was conducted by BARC with the assistance of the Bureau of Economics and Statistics, Kerala state and the results were published in 1972. Details of 13,720 pregnancies were obtained by interviewing 2420 couples living in areas spread over the full length of the radioactive strip. The questions sought information relating to the following indicators of genetic change: sex ratio among the offspring, fertility index, infant mortality, pregnancy terminations, multiple births and gross abnormalities**.**

Differences in the fertility index between groups receiving different radiation levels were not found to be statistically significant although there was a tendency towards lower values as the dose levels increased. However, the extremely small sample size of 22 couples in the highest exposure group (> 20 mGy/yr ) made it difficult to draw any firm conclusion.

A field laboratory was set up by BARC in 1975 at Neendakara (Monazite Survey Project). Research activities initiated in this laboratory include:

• Cytogenetic studies to estimate frequency of chromosome aberrations and karyotype anomalies among newborns.

## Gene-environment interaction




*The factors that can have an individual/interactive effect. Delineating radiation effects (from background effects) after taking into account the other confounding/competing factors is a daunting task and requires large scale studies*

• Newborn monitoring programme to identify major congenital malformations.

• A health audit survey to provide general disease profile in the population including prevalent malformations and late onset diseases with the sociodemographic characteristics of the population under study.

• Estimation of germinal mutation rate in the families using DNA markers such as minisatellites and microsatellites.

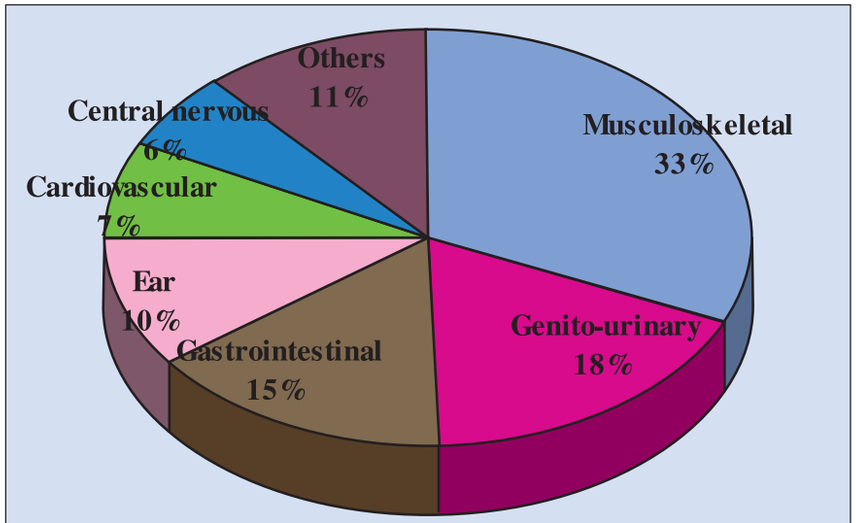
• A dosimetric survey employing Solid State Nuclear Track Detector (SSNTD) and Thermo Luminescent Dosimeter (TLD) to evolve a dosimetric map of the entire HLNRA.

Such studies would provide a realistic view of gene environment interaction in human population and health and biological consequences of continuous radiation exposure after consideration of the various confounding factors.

So far, over 90,000 newborns have been monitored for congenital malformations. The overall incidence of malformations is 1.98% and the main systems involved are musculo-skeletal followed by genitourinary, gastrointestinal, cardiovascular and central nervous system. Congenital malformation showed maternal age dependent increase. Frequency of malformation and stillbirths did not show any correlation with radiation levels after taking into account all the possible confounding factors.

This newborn screening programme is still being continued. The Board of Research in Nuclear Sciences (BRNS) sponsored two projects on the evaluation of the rate of congenital malformation in normal radiation background areas, one in newborn children in Mumbai, Vadodara and New Delhi and the other in Chennai. In the first study nearly 96,000 newborns were monitored. The Chennai study monitored 72, 000 newborns. Incidence of congenital malformation in these two studies was similar to that observed in the HLNRA of Kerala.

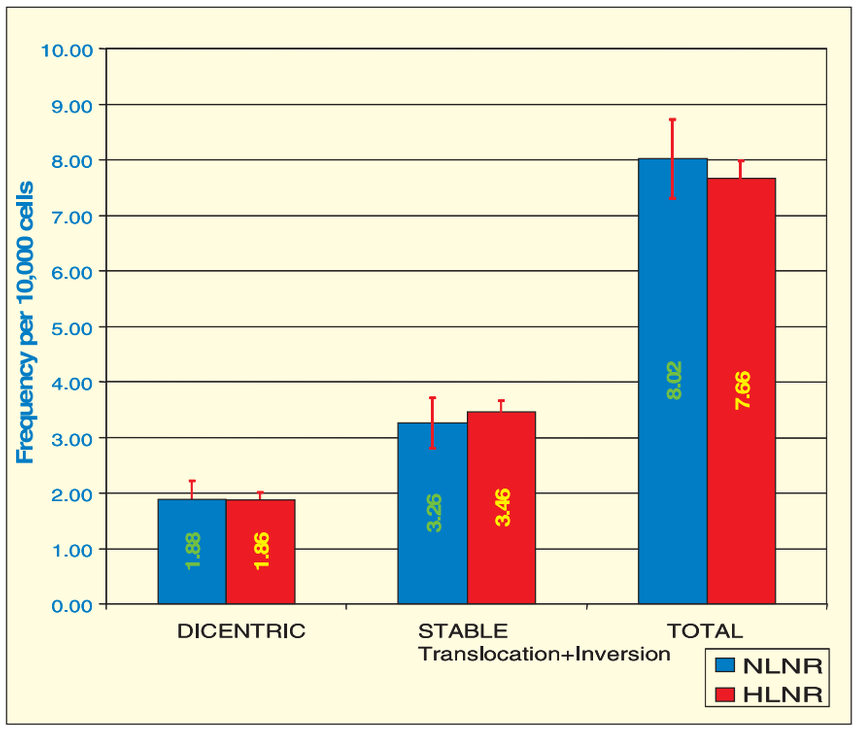
Karyotype analysis of over 24,000 cord blood samples from normal as well as high background areas was carried out during the past 15 years. The overall incidence of constitutional




*New born survey. System-wise split-up of observed malformations*

karyotype anomalies from normal as well as high background areas was 4.85±0.45 per 1,000 newborns which is in agreement with data published from other parts of the world. Numerical anomalies were more among males whereas structural anomalies were more among females. There was no significant difference in this frequency among samples screened from HLNRA and NLNRA.

The hypervariable regions of human DNA are represented by minisatellites and microsatellites which show much higher rate of spontaneous mutation. In an effort to assess the genetic effect of this chronic exposure at the DNA level, over 200 families were analysed using 50 DNA markers (loci) including 10 minisatellites and 40 microsatellites. Data collected so far do not show any change in germinal mutation rate in HLNRA compared to NLNRA.




*Frequencies of chromosomal aberrations in blood lymphocytes collected from NLNR and HLNR*

A house-to-house comprehensive Health Audit Survey was completed in three Panchayats in the study area to assess the pattern of chronic/late-onset diseases/disorders, congenital malformations and untoward pregnancy outcome in married women in the age group of 15-49 years with respect to socio-demographic profile, life style and background natural radiation levels. Similar survey will be carried out in the remaining Panchayats.

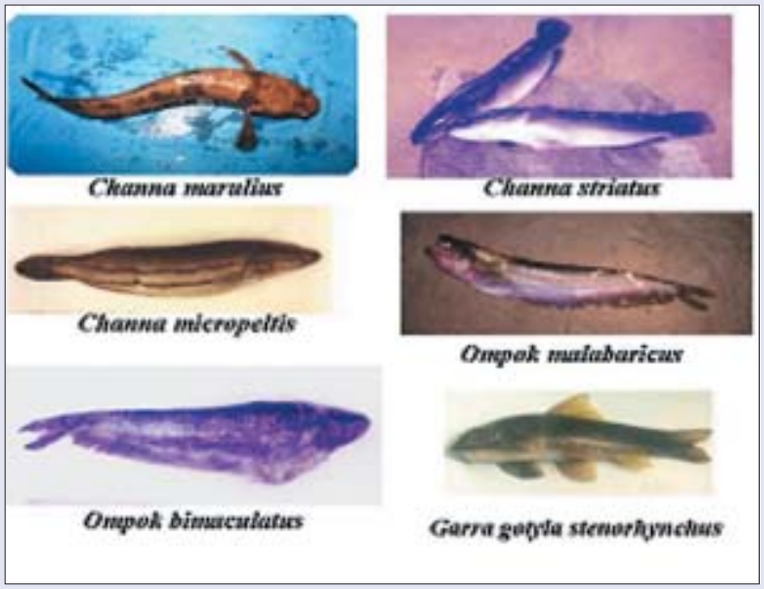
A Natural Background Cancer Registry project supported by the Board of Research in Nuclear Sciences was initiated with the prime objective of studying occurrence of cancer vis-à-vis chronic natural radiation exposure in the population of the Karunagapally Taluk of Kollam district. During 1990 to 1998, 3,59,619 persons were enumerated and socio-demographic information was collected by face to face interview by trained social investigators. Prevalence of cancer and associated factors was assessed in the population. Radiation dose measurements were recorded outside 76,942 houses and inside 71,674 houses. Till the end of 1997, 2567 cancer cases were identified giving age standardized incidence rates of 104.6 for males and 76.6 for females per 100,000 population. The total cancer incidence did not show increase with radiation level in both males and females. Though lung cancer in males showed a slight increase at high radiation level, no statistically significant dose-effect relationship was observed. Further, there was no increase in lung cancer in females. Contribution of confounding factors like smoking and life styles needs to be critically evaluated. The number of specific cancer cases is too small to reach any firm conclusions. Incidentally, this is only the second rural cancer registry in India.

# Ecological Studies and Bioremediation

## Link B12

*Prosperity of a nation can be judged from its per capita electric power consumption. Every kind of industrial development, nonetheless, apart from the benefit it offers in terms of wealth, has its costs. Electric power generation is no exception. The costs include both the financial burden as well as the ecological impact. In recent years, appreciation of such ecological/environmental costs is, slowly but surely, increasing all over the world. Electric power plants are often strategically located in close proximity of natural water bodies such as lakes, rivers, estuaries and oceans. They use such water bodies as heat sinks. Thermal discharge into water bodies is a major concern, both for power producers and especially for environmental regulatory agencies, such as the Ministry of Environment and Forests (MOE&F) and Pollution Control Boards (PCBs) in our country. Electric power production can lead to increase in temperatures due to (a) a direct discharge of heated water, and (b) indirect global warming through $CO_2$ and other green house gases (GHGs). Of these, dissipation of heat through the plant cooling water systems is the only thermal discharge from nuclear power plants. Ecological survey of water bodies around power plants is an important endeavor to assess the biological impact of thermal effluents. Other concerns being actively addressed to in nuclear power production programme are bioremediation of nuclear wastes, both organic and inorganic.*

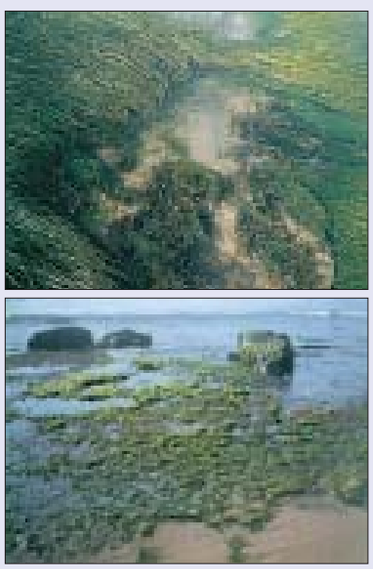
# Zooplankton diversity in the vicinity of various nuclear power plants





*Images of fish population diversity in Kunakulam*

*At the nuclear power plant under construction at Kudankulam efforts have been initiated to seek baseline ecological information about physicochemical parameters of water quality, nutrient status and occurrence of major flora and fauna at the site*



*Primary producers at Kudankulam*

*Typical fish catch at Kudankulam*

*Fish catch in the vicinity of MAPS, Kalpakkam*

# Ecological Studies and Bioremediation

In recent years special projects have been sponsored by DAE to study problems associated with the use of natural waters as industrial coolants, along with associated ecological and operational problems. Environment-related work includes thermal ecology studies in the vicinity of the power stations and bioremediation of toxic wastes. Most life forms (mesophiles) show optimum temperature requirement in the range of 25°C-30°C. Even so, they tolerate temperature rise up to 40°C or 42°C. Temperatures above 45°C are generally lethal. Most life-forms are equipped to deal with impairment caused by moderate temperature upshifts (5°C-15°C) of short durations (from minutes to few hours). Many life-forms, such as bacteria, algae, fungi, insects, plants and animals were investigated in laboratories for their tolerance to high temperatures. But such "knowledge" based on few model organisms neither allows us to predict thermal behavior of other "similar" organisms nor does it permit extrapolation of findings to field ecosystems since they tend to be highly heterogeneous, location-specific and subject to seasonal variations. Also, intricate relationships between different organisms in a food chain complicate prediction of thermal response of the entire ecosystem.

Experience in temperate climates has shown little ecological effect due to heated effluent discharge. Thermal pollution of water bodies does not seem to be a cause of serious concern in those areas. It would, therefore, appear that most organisms in tropical water bodies could tolerate a temperature change caused by the difference of 5 to 10°C provided the temperature of the water body does not increase above 35°C. However, the major El Nino/ocean warming experiences in 1998 demonstrated ill effects of thermal pollution on the aquatic ecosystems like bleaching of coral reeves. The new pollution control legislation stipulated a new limit for $\Delta T$ not greater than 5°C which created a serious problem for power plant operators.

In order that a realistic assessment of the biological impact of thermal discharge under Indian conditions can be made, DAE took the initiative in setting up the first major thermal ecological study in the country. Eight laboratories are participating in this study. Careful monitoring of thermal plumes for two years at Kalpakkam and Kaiga showed that these plants have been operating in total compliance with mandatory requirements of thermal discharge. Clear indications are now available on the biological impact of heated water effluents at Kalpakkam and Kaiga. At both sites, the general abundance of biota decreases right at the outfall but is restored to near normal within a short distance from the discharge point. Both the qualitative and quantitative changes are being carefully documented. It is expected that such data would help to clearly define a "mixing zone". Various indicator organisms were exposed to temperature ranging from 27 to 42°C in laboratory experiments. Impact of thermal and chemical (chlorine) stress on different organism groups was also studied. Response of phytoplankton was evaluated in terms of chlorophyll loss as well as change in primary production. A new programme is initiated to study the distribution of chlorination byproducts (such as trihalomethanes) in the vicinity of the power station's discharge. An experimental seawater mesocosm facility was built, commissioned and used to study the population level impact of thermal stress on phytoplankton and phytobenthos.

## Biofouling and biocorrosion

Under operational problems of costal power plants, the major concerns are biofouling (marine growth) in the condenser cooling circuits of coastal power plants, condenser slime formation, scaling and biocorrosion in cooling water circuits.

The biology group of Water and Steam Chemistry Laboratory, Kalpakkam established in early 1980, is engaged in marine and fresh water research with a view to study the problems associated with the use of natural waters as industrial coolants, operational and ecological problems related to above aspect. After a careful study of the biofouling problem, a chlorination regime based on continuous low dose application has been recommended and is being practiced at Madras Atomic Power Station (MAPS). Extensive data were also generated on biological aspects of the major fouling species of mussels and barnacles and their response to chlorination. Another achievement was the development and standardization of a larval culture and bioassay protocol using *Balanus reticulatus*, a fouling barnacle. In addition, a circulating seawater facility, called the Biofouling Test Loop Facility has been designed, built and commissioned, with which biofouling and biocorrosion could be studied under controlled conditions.

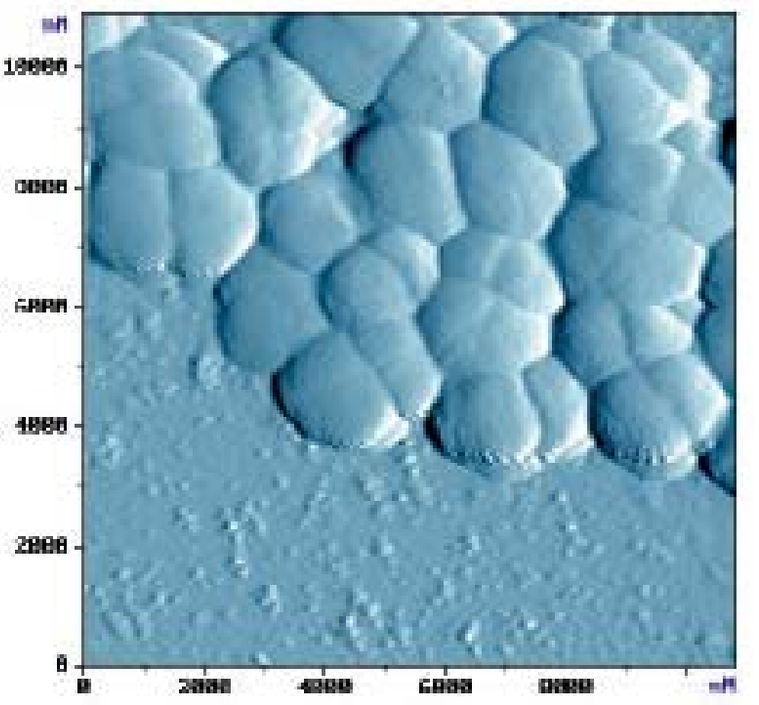
The service water system of reactors encounters extensive metal loss and pipe plugging due to corrosion in its carbon steel lines. This is caused by iron oxidizing and sulphate reducing bacteria, which were shown to be responsible for extensive pitting and tubercle formation inside the service water system. Similarly, cracking of brass condenser was due to nitrate reducing bacteria present in the condenser slime. Recent work at Kalpakkam showed that titanium, generally known to be resistant to pitting type of corrosion, can also be attacked by sulphate reducing bacteria.

## Bioremediation

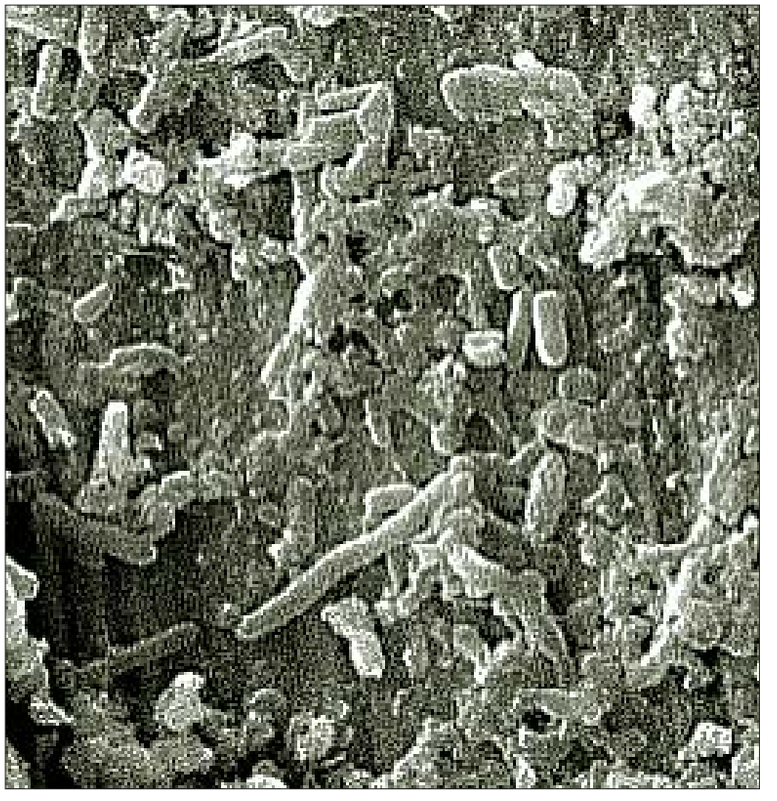
Bioremediation is important to DAE's ambitious nuclear power programme and the envisaged treatment of high volumes of radioactive wastes. Mobility of heavy metals, radionuclides and other toxicants into aquatic and terrestrial environment by mining, industrial processes and municipal waste deserves significant scientific, public health and regulatory attention. Bioremediation is emerging as one of the several alternative technologies for removing toxic wastes from environment. Today, it is becoming a technically viable option, based on good science and engineering. Common wastes generated during a nuclear cycle include, among others, uranium, thorium, radium, plutonium, cesium, strontium, ruthenium, zirconium, technetium, complexing agents such as EDTA, NTA, citric acid, sulphates, nitrates, carbonates and tributyl phosphate.

Bearing in mind the fact that biofilms have tremendous biotechnological applications, work was initiated on a few aspects related to bioremediation. Bacteria capable of degradation of complexing agents (EDTA, NTA) were isolated from sewage. Manganese oxidizing bacteria can scavenge a variety of metals and radionuclides. Biofilm formation by radiation resistant *Dienococcus radiodurans* on various material surfaces was studied using a variety of techniques, including atomic force microscopy. Experiments were carried out with labeled (using fluorescent proteins) bacteria on horizontal gene transfer in biofilm environments.

Studies at BARC have identified certain bacteria and fungi, which are potential biosorbents of radionuclides such as U, Cu and Th . These include fungi like *Aspergillus fumigatus, Rhizopus arrhizus* and *Rhizopus oryzae* and bacteria like *Pseudomonas*



The fine structure of *Deinococcus* biofilm observed with atomic force microscope



*SEM picture showing long rod shaped sulphate reducing bacteria on titanium*

*sp*. Some agarose based biosorbents were also studied. Promising among them are the muciligenous seeds and dried roots of water hyacinth. Extracellular polysaccharide (EPS) of a *Pseudomonas aeruginosa* strain was also shown to be a potential biosorbent of Cu, Th and U. A variety of techniques developed earlier for immobilization of cells were applied for the pelletisation of the microbial biomass for use in continuous bioreactors. A process for the bulk production of biobeads/ bioresins has been scaled up. One such beaded biomass (BARC Bioresin) was successfully used for on-line decontamination of $^{60}Co$ containing liquid waste (pool water) under realistic conditions. This is one of the first few studies demonstrating the possibility of using a biosorbent for treatment of radioactive wastes.

Phytoremediation is also gaining importance as a means for cleaning up of contaminated soils as well as liquid wastes through the process of rhizoextraction, rhizofiltration and biodegradation. Inherent capability of plants to take up essential elements through roots can be manipulated for bioaccumulation of toxic heavy metals and radionuclides. Studies, therefore, were initiated on some of the hyper-accumulator/ metal tolerant plants like the Indian mustard, sunflower, *Sesbania*, tomato, vetiver etc. Protocols were established for production of hairy roots in these plants. Accumulation of metals/radionuclides by such hairy roots will be examined using live and dead biomass.

### Use of biotechnology for bioremediation

In recent years, a transgenic *E. coli* expressing the acid phosphatase gene *phoN* from *Salmonella typhimurium* has been constructed which facilitates bioprecipitation of uranium from low level radioactive waste. The gene has now been moved to and expressed in *Deinococcus radiodurans* to allow use of radio-resistant live cells of this bacterium for bio-precipitation.

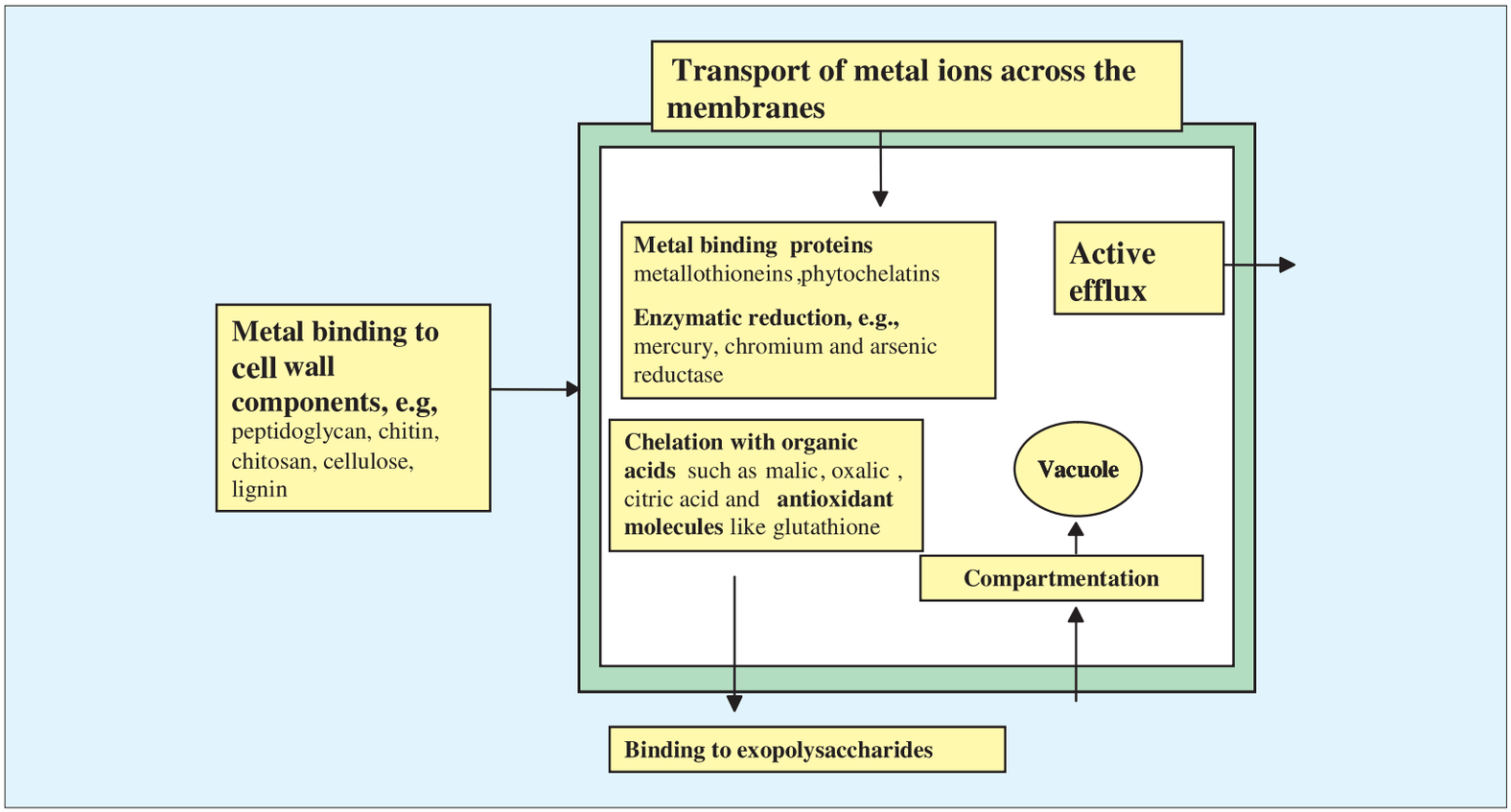
**Some constituents of nuclear wastes and processes involved in generation of these wastes**

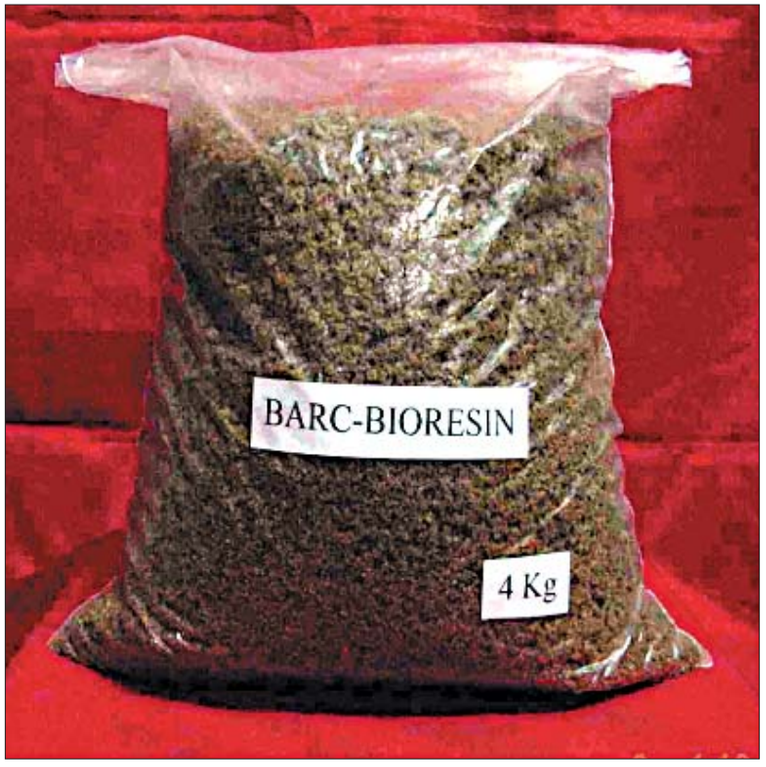
| | | |
|---|---|---|
| Uranium | - | Mining, fuel fabrication and reprocessing |
| Thorium | - | Mining and fuel fabrication |
| Radium | - | Low active waste difficult to treat by current methods |
| Plutonium | - | Nuclear fuel reprocessing ($^{241}Pu$ – beta emitter short lived and $^{239}Pu$, $^{240}Pu$, $^{242}Pu$ – alpha emitters and persistent) |
| Cesium & Strontium | - | Fission products (highly active, long lived) |
| Ruthenium | - | Fission product regarded as trouble-some in discharges |
| Zirconium | - | Fission product, long half life and from dissolution of fuel claddings |
| Technetium | - | Fission product, long half life, mobile |
| Sulfate | - | High levels in reprocessing wastes |
| Nitrate | - | High levels in both fuel fabrication and reprocessing wastes |
| EDTA, DPTA, NTA & CITRATE | - | Deliberately added to decontaminate active systems, forms very strong complexes with actinides and promote their migration in environment |
| Carnbonates | - | Form strong actinide complexes |
| Tri-Butyl Phosphate | - | Extraction of U and in separation of U and Pu during fuel reprocessing |

**Bacteria have taste for waste**

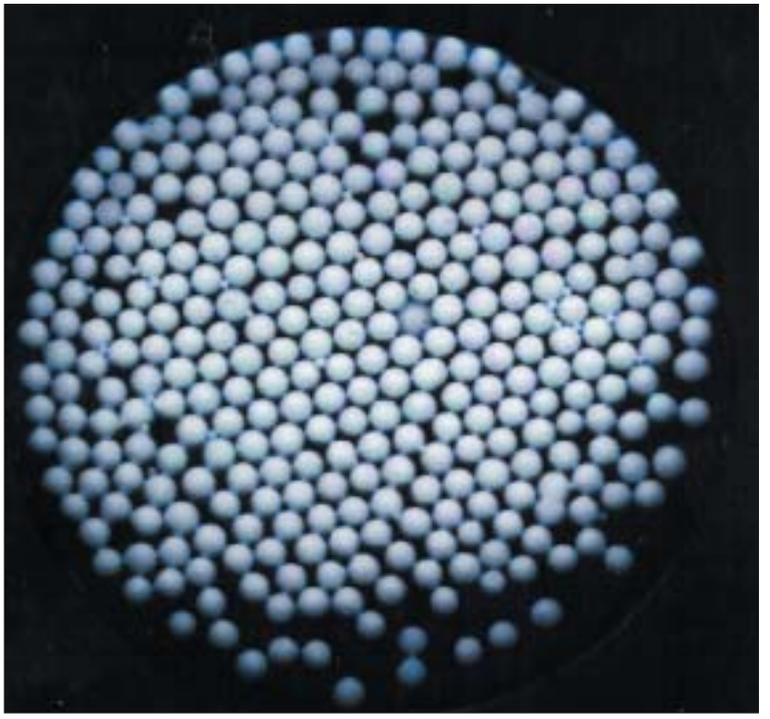
- Iron Oxidising Bacteria: *Thiobacillus, Leptothrix, Gallionella, Sphaerotilus*
- Iron Reducing Bacteria: *Pseudomonas*
- Nitrate Reducing Bacteria: *Nitrosomonas, Nitrosobacter. Nitrosococcus*
- Exopolymer Producing Bacteria: *Pseudomonas aeruginosa*
- Sulfate Reducing Bacteria: *Desulfovibrio vulgaris*
- EDTA and NTA Degrading Bacteria: *Chelatobactor, Pseudomonas*
- Radiation Resistant Bacteria: *Deinococcus radiodurans*

## Cellular interaction of toxic metal ions and tolerance mechanisms




*Beeded biomass (BARC-bioresin) used for on-line decontamination of $^{60}Co$ containing liquid waste*



*Immobilized dead cells for bioremediation biobeads: Pseudomonas cells immobilized in polyacrylamide beads polymerised by gamma rays*