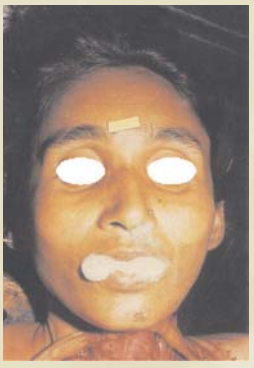
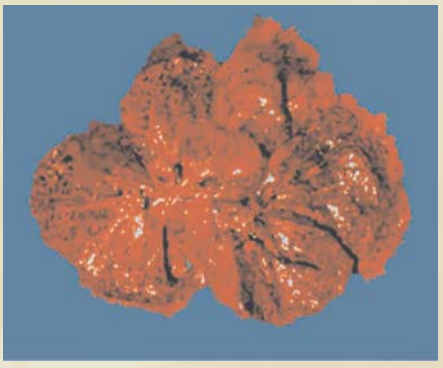
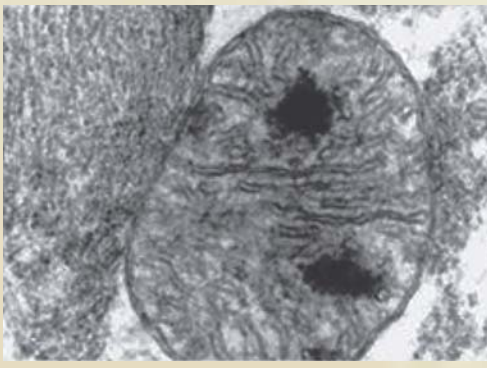
HEALTH EFFECTS OF THE TOXIC GAS LEAK FROM UNION CARBIDE METHYL ISOCYANATE PLANT IN BHOPAL

TECHNICAL REPORT

ON

Pathology and Toxicology

(1984-1992)

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(INDIAN COUNCIL MEDICAL RESEARCH)

NEW DELHI

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Ansari Nagar, New Delhi - 110 029 (INDIA)

2010

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2010

Published by
Director-General
Indian Council of Medical Researach
Department of Health Research
New Delhi- 110 029

October 2010

Production Controller
J.N. Mathur, Press Manager; ICMR, New Delhi

Printed at: M/s Royal Offset Printers, A-89/1, Naraina Industrial Area, Phase-I, New Delhi-110 028

सत्यमेव जयते

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सचिव, भारत सरकार
(स्वास्थ्य अनुसंधान विभाग)
स्वास्थ्य एवं परिवार कल्याण मंत्रालय एवं
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Preface

THE largest Chemical Disaster in the history of the World viz., Bhopal Gas Disaster (BGD) occurred on the fateful night of 3rd December, 1984. The helpless doctors were neither aware about the nature of 'Killer Gas', which had escaped into the air from the Union Carbide India Ltd (UCIL) factory, nor did they have any idea about antidotes to be administered. Yet, they did whatever they could by way of symptomatic treatment, to make the last minutes of the victims on earth, more tolerable. The nature of the 'Killer Gas' that descended on the unfortunate citizens of Bhopal on that fateful night and the appropriate treatment was eventually established.

Several thousands of families had to be helped to recover from the "**trauma of the disaster**" viz., the loss of their dear and near ones, loss of cattle and urgent need for rehabilitation. The Medical Community, of Bhopal in particular and the country at large, rose to the occasion and met the challenges posed by the grave threat to human and animal life and the long-term and persistent effects in the survivors. Soon, the Indian Council of Medical Research (ICMR) organised teams of Indian Scientists to grapple with each one of the above problems. Last but not the least, the Director General of ICMR, Prof. V Ramalingaswami, acted as a catalyst and evinced keen interest in the promotion of trans-disciplinary multi-institutional scientific collaboration. As a result, concerted efforts including autopsies, toxicological studies, clinical management of the victims and epidemiological investigations with many significant results had been obtained and had helped to unravel some of the mysteries of this major industrial disaster.

Late Prof. Heeresh Chandra, Director of the unique Medico Legal Institute, MG Medical College of Bhopal, along with Dr. Satapathy & his faculty, undertook massive and thorough Forensic Autopsy studies, as per international standards. Virtually starting from Day One of BGT, a total of 731 bodies were studied during the month of December, 1984 itself and thereafter as and when there were suspected casualties. Due to the unprecedented rush in the first three days, while detailed External Findings were recorded in 337 out of the 620 bodies received, complete autopsy was done in the 283 cases, followed by 111 cases later on in December. Initially, he was assisted by his entire staff of MLI and Dr. Darbari & Dr. Kanhere of Dept of Pathology of MGMC. From the 12th of Dec '84, they were joined by Late Dr. S Sriramachari & Dr. HMK Saxena of Institute of Pathology of ICMR at New Delhi. The latter team took over Histopathological studies of the entire range, including Electron Microscopy and Experimental studies carried out by Dr. K Jeevaratnam of DRDE, Gwalior. The MLI, entrusted with "ICMR Project – 08", concentrated on several contentious Toxicological issues like Recurrent Cyanide Toxicity, NaTS therapy and over 18,000 samples of Urinary Thiocyanate (SCN^-) levels , reduced blood TNBS levels due to MIC cross-over of 'Alveolar Capillary Barrier', foretelling the most probable Biochemical Lesion of S-Carbamoylation of Blood & Tissue constituents by MIC, Blood Gas Analyses, related Pulmonary Physiology, etc. An attempt was also made out to explore potential 'Cyanogenic Nitriles' from amongst the particulate components of Tank Residues, inhaled by the victims. In the ultimate analysis, drawing upon the 1982 paper on thermal decomposition of MIC by Blake and

Izadi Maghsoodi, the riddle of both the Acute & Fatal as well as delayed Recurrent Cyanide Toxicity has been resolved, based on incontrovertible data on blood cyanide levels, urinary thiocyanate levels and N-Carbamoylation, gathered in the Project.

These investigations were undertaken under the joint guidance of Prof. Heeresh Chandra, Dr. S Sriramachari and a host of co-investigators like Prof. A Ramaiah (AIIMS), Prof. PS Narayanan (GB Pant Hosp), Dr. PK Ramachandran & Associates of DRDE, Gwalior and DIPAS, New Delhi, etc. Apart from the above participants, a major share of the credit should go to the enthusiastic scientific staff recruited on the project 08, especially Dr. GJ Rao and Dr. Arun Saraf, but for whose dedicated and commitment to the work, it would have been impossible to attain scientific success.

Initially, there were official restrictions about publication of the scientific results since the matter was *sub-judice* in USA & India. Later on several scientific agencies started exercising relaxations, although the ICMR became the butt of criticism for this delay in publication of longterm followup. Slowly but gradually, its findings were presented in different National & International fora.

ICMR has already brought out two Technical Reports pertaining to "Health Effects of the Toxic Gas Leak from the Union Carbide Methyl IsoCynate Plant Bhopal". The First is based on Population Based "Long Term Epidemiological Studies"and the Second onto "Long Term Clinical Studies". They pertain to two major sets of ICMR Research Projects undertaken during the period 1985-94 and are in the nature of compilation of all the relevant information, including publications and relevant bibliography. There has been some unavoidable delay in the complication of the third and last of the Bhopal Technical Report Series related to "Pathology and Toxicoloy", due to periodical sickness of even the few limited investigators familiar with ICMR Projects-08.

In view of the enormous quantum of data, it has been decided to present it in a series of Sections or Chapters. Since much of it is contained in relevant publications, and with a view to avoid repetition, first under each section an overview is presented, outlining the issue(s) encountered and the manner in which it had been resolved, followed by a series of Annexure's comprising relevant publications by the team members. Thereby the entire Report will be in the nature of a true compendium of the work done under Project-08. I would like to suggest to readers to study 1987 special supplement of Indian Journal of Medical Research (IJMR), on this subject paper of Current Science (Vol 86, No 7, 10th April 2004, pp 905-920) and other two technical reports alongwith so as to get a comprehensive picture.

In spite of personal problems, as one associated with most of the inter-related Bhopal investigations from their inception, Dr. S Sriramachari, INSA Honorary Scientist (formerly Addl. DG-ICMR), was able to complete the task assigned to him. This volume is being presented as a tribute to this illustrious person and a great scientist. He had earlier published most of the important findings in Current Science in 2004. Even draft for this "Preface"was prepared by him before his death.

(Dr. V. M. KATOCH)

Acknowledgements

THE project **"Population Based Long Term Clinical Studies on Health Effects of Bhopal Toxic Gas Exposure"** has been conducted for almost a decade (1985-1994) and a large number of scientists from different parts of the country have contributed to these studies. Hence, our utmost thanks are due to all these scientists.

Thanks are due to Late Dr. V Ramalingaswami, Late Dr. AS Paintal, Dr. SP Tripathi, Dr. GV Sathyavati & Dr. NK Ganguly, Ex Director Generals; Late Dr. S Sriramachari, Dr. Usha K Luthra, Dr. Padam Singh, Ex. Additional Director Generals; Late Dr. CR Ramachandran, Ex. Sr. Deputy Director General; Late Dr. AK Prabhakar, Ex-Deputy Director General; Dr. Rashmi Parhee, Ex-SRO, Officers of NCD Division and other members of the Indian Council of Medical Research for Technical Guidance; and Ministry of Health & Family Welfare for the prompt financial support. The Council wishes to place on record its sincere thanks to Shri Tanwant Singh Keer, Ex-Minister, Bhopal Gas Relief and Rehabilitation Department, Govt. of Madhya Pradesh, Shri Ishwar Das, Shri S Satyam and Shri CS Chadha, the then Principal Secretaries, Govt. of MP Department of Bhopal Gas Relief for enabling such a major activity to be successfully carried out. Dr. PK Bhat, the then Director, Centre for Rehabilitation Studies provided valuable support and cooperation in the preparation of this report. I wish to place on record my sincere thanks to all the team members for their willing cooperation and for their very hard work.

My special tribute to the following departed souls who had contributed largely to our joint research endeavours in Bhopal: Prof. P. S. Narayanan, Prof. Heeresh Chandra, Dr M. P. Dwivedi, Dr. C. R. Ramachandran, Prof. V. Ramalingaswami and Dr. C. R. Krishnamurthi.

Special thanks are due to Dr. S. Sriramachari, Dr. K. Satyanarayana, Dr. A.K. Jain and Dr. S.K. Jain for facilitating the publication of this Report and to Dr. D.K. Shukla, Scientist-F, NCD, Division, ICMR for assisting them and pursuing the progress of this Report. Thanks are also due to the secretarial staff of BGDRC and secretarial staff (Mr. Ravi Deval, Miss Manju, Mrs. Kamlesh) attached to Late Dr. S. Sriramachari for typing the manuscript of this report.

Our sincere gratitude is due to all the people who extended their cooperation and time for participation in this study over a protracted period of time.

(Bela Shah)
Scientist G & Head
NCD Division

Contents

Abbreviation Used

2-3 DPG	2-3 Di-Phospho Glycerate
amu	Atomic Mass Unit
ARDS	Acute Respiratory Distress Syndrome
BGD / T	Bhopal Gas Disaster / Tragedy
BGDRC	Bhopal Gas Disaster Research Centre
DBCT	Double Blind Clinical Trial
DBCTs	Double Blind Clinical Trials
DIPAS	Defense Institute of Physiology and Allied Sciences
DMIT	Dimethyl iso-Cyanurate
DMU	Di-Methyl Urea
DRDE	Defense Research Development Establishment
GBPH	Govind Ballabh Pant Hospital
GLT	Glass Lined Tube
Hb	Hemoglobin
HCN	Hydrogen Cyanide
ICMR	Indian Council of Medical Research
INMAS	Institute of Nuclear Medicine and Allied Sciences
IOP	Institute of Pathology
IVRI	Indian Veterinary Research Institute
m/z	Mass to Charge Ratio
MA	Methyl Amine
MGMC	Mahatma Gandhi Medical College
MIC	Methyl Iso-Cyanate
MLI	Medico Legal Institute
NaTS	Sodium Thio Sulphate
TCA	Trichloro-Acetic Acid
TNBS	Tri-Nitro-Benzene- Sulphonic acid
TRC	Tank Residue Constituents
UCC	Union Carbide Commission
UCIL	Union Carbide India Limited

CHAPTER

1 INTRODUCTION: BHOPAL GAS DISASTER

THE Bhopal Gas Disaster, of 2nd-3rd December, 1984, caused by a "run-away chemical reaction" of Methyl IsoCyanate stored in a Stainless Steel Tank, of UCIL (Union Carbide of India Ltd) Factory, is undoubtedly the worst chemical disaster of the world. The sheer magnitude of the industrial catastrophe has aroused the conscience of the world. The incriminated Tank 610 E of the 'Pesticide Plant' of Union Carbide Corporation (UCC) in Bhopal was maintained by its Indian counterpart, Union Carbide India Limited (UCIL). Unlike minor accidents in their US Plants, immediately following the chemical run-away reaction of Methyl IsoCyanate (MIC) in Bhopal, there was a massive release of toxic gases into the atmosphere which spread rapidly over a densely populated area of Bhopal City, the capital of Madhya Pradesh, India.

Premonitory Lapses: Several adverse factors have been implicated in this disaster, including prolonged bulk storage (42 tons) of MIC, a non-functioning Refrigeration System, and above all, the failure of basic safety measures, like regular tracking of Pressure & Temperature of each Tank and non-functioning or inadequate facilities for neutralisation of a highly reactive chemical product. Above all, the accident seems to have been precipitated by a sudden influx of water through a mal-functioning valvular device! One or more of these factors seem to have contributed to the uncontrolled accidental runaway reaction, as indicated in the Official Report of Varadarajan and associates (1985). Similarly, based on a critical "Analysis of the Bhopal Accident", in a paper published in 1987, Bowonder suggested that the Bhopal accident is due to a series of errors, not only of the 'hardware', but equally serious lapses which fall under "operator, information and systemic error categories". Finally, according to him, "the most critical reason" for the occurrence of these errors is due to corporate level "failure of safety management systems and procedures (Bowonder, 1987).

Human Mortality & Morbidity: By all accounts, the Bhopal Gas Tragedy took a sudden and heavy toll of human (and animal) lives. People started dying within hours; by 7.00 AM, 70 people were dead, by 9.00 AM 260 were dead and thereafter the figures continued to rise. Although all the dead bodies may not have reached the morgue of MLI of Bhopal, 311 bodies were received on December 3, 1984, followed by another 250 on December 4, 1984; thereafter, the rate declined. A total of 731 bodies were received in December 1984 alone, 103 in 1985; 90 in 1986 and 44 and 22 respectively in 1987 and 1988. These figures from the morgue may not account for all the deaths in the city of Bhopal. The over-all Government estimates placed the number of dead at around 1900, whereas on a conservative estimate more than 2000 lives were lost in the first few days.

The loss of human life and prolonged incapacitation of many survivors is unprecedented. In many cases, the entire family or the sole bread earner died; several were widowed; and many of the survivors were either infants or minor children. For several weeks and months, large number of survivors exhibited a wide range of respiratory, ocular, behavioural and other morbidities. In subsequent years the MLI continued to perform autopsies on gas-affected victims, although markedly reduced in numbers. Certain pertinent doubts remained, as to how long the symptoms would last and whether the ocular manifestations of more than 90% would lead to serious visual impairment.

Medical Relief and Research: The prompt relief measures, initiated by the local health authorities of the MP Govt., were soon supplemented by research investigations on a massive scale by several scientific organizations, notably by ICMR, since it is primarily responsible for Medical Research in India. It drew upon its readily available multi-disciplinary scientific manpower across the country and rapidly mobilised requisite resources and scientific expertise to tackle every conceivable line of investigation. With a view to facilitate equally appropriate and scientifically rational therapeutic relief to the victims, issues related to Pathology & Toxicology were given immediate attention, alongside Clinical & Reproductive Issues, even before initiating extensive studies related to Epidemiology and new fields like Clinical

Psychology and Genomics. Therefore immediate attention could be bestowed to the highly controversial issue of 'Cyanide Toxicity, the possible cause of immediate deaths and the need for timely therapeutic intervention based on DBCT with NaTS, coupled with extensive monitoring of Urinary Thiocyanate levels.

In the process, the ICMR instituted 24 major Research Projects, ranging from Epidemiology to Molecular Biology and distributed in 15 National institutions, including several of its own, both scientifically and financially, and drawing upon the scientific expertise of 24 Principal Investigators and over hundred other scientists, engaged in a nation-wide research effort on the health consequences of the Bhopal Gas Tragedy. It also established a short-term Bhopal Gas Disaster Research Centre (BGDRC) and allocated about Six Crores of Rupees for its above ventures.

Scientific Issues: The total unavailability of prior knowledge about MIC Toxicity and detoxification / therapeutic intervention was a great handicap and added to the confusion. It is worth recalling that immediately after the Disaster, even the UCC had no positive information either about the toxic effects of MIC or of any antidotes. However, it unleashed a subtle campaign that on contact with the aqueous surfaces of the airways and even before it crosses the airways, MIC breaks down into relatively harmless compounds, like Methyl Ami2ne & Di-Methyl Urea. From the beginning, a majority of ICMR scientists were not convinced of the hypothesis of UCC. Instead, they undertook a trans-disciplinary "Interventional Clinical Toxicology". The efficacy of NaTS therapy was first established, followed by proof of delayed / recurrent 'Cyanide Toxicity', independent of the issue of initial liberation of HCN.

The portentous and pioneering approach on the Kinetics & Mechanism of the Thermal Decomposition of Methyl Isocyanate by Blake & Ijadi-Maghsoodi published in 1982, two years before BGD, was a virtual premonition of the Bhopal Tragedy. Fortuitously, the Toxicology Team of ICMR, in the teeth of opposition early in 1985, stumbled upon this illustrious publication, which has provided a rational scientific basis for timely interpretation of early deaths in BGD, a unique and unprecedented global event. Otherwise, it would have been lost in the limbo of scientific oblivion and NaTS therapy remained empirical.

Eventually, MIC and its derivatives were also traced in the blood stream and several organs and tissues of the body of victims and even survivors exposed to the Toxic Aerosol of Bhopal. Apart from establishing an entirely new phenomenon of irreversible N-Carbamoylation of end-terminal Valine residues of Hb, the nature of impaired tissue oxidation and step-up of 'alternate respiratory pathways' such as transient elevated 2-3 DPG levels of blood has been demonstrated. Simultaneous N-Carbamoylation of several other end-terminal amino acids of tissues proteins confirmed the wide-spread dissemination of MIC in the body, contrary to UCC's postulations. In spite of the finding of reduced levels of blood Glutathione the crucial issue of S-Carbamoylation could not be achieved due to lack of proper equipment. Otherwise, it might have been possible to link up MIC with 'impaired cyanide toxicity' through the reversible phenomenon of S-Carbamoylation. It is a matter for some consolation and comfort that eventually the work on *in vivo* transport of MIC by Bailie and Slatter (1991) lends theoretical support to the role of MIC in the issue of chronic & recurrent cyanide toxicity advocated by us in Bhopal. Lastly, even at the initial stages of the investigations, it was suggested that the toxic gas which leaked in Bhopal contained not only MIC, stored at ambient temperature, but also several other chemicals generated at the higher temperatures of the runaway reaction. Since amongst them there could be potential CN-yielding Nitrile compounds, a thorough study of Tank Residue Compounds was also undertaken as part of the Cyanide Studies.

Long Term Clinico-Pathological Studies: Apart from Autopsy and Histopathological studies, Clinical, Forensic & Experimental Toxicology were undertaken by the respective ICMR teams and their associates. The collaborative research efforts continued for over a decade. Several hitherto unknown aspects were successfully investigated and quite a few toxicological dilemmas of Bhopal tragedy were unravelled. In this Report an attempt has been made to briefly highlight the full gamut of the findings, both published and unpublished. The periodic presentations on Forensic & Histopathological aspects by Heeresh Chandra and Sriramachari at several National and International forums evoked great attention.

It is often said that "the dead teach the living" and probably it is all the more true in the case of the Bhopal tragedy. Indeed a large part of the credit probably should go to Late Prof. Heeresh Chandra whose indomitable will and tenacity was a source of inspiration to all the investigators and a great hope for the surviving victims. It must be recognised that while the eyes and respiratory system showed striking disturbances from the beginning, widespread

multi-organ involvement in the exposed population was also observed. There were cases of coma, a striking feature in the acute phase; gastrointestinal disturbances were common; there were significant lesions in the central nervous system; above all psychological trauma and behavioural disturbances continued to be a dominant feature without abatement to this day for a long time. Both sexes and at all age groups from in-utero conception to old age, were affected. There were early indications of immunological disturbances with as yet unforeseen effects on host susceptibility to environmental infections and other hazards; chromosomal abnormalities were noted in the early acute phase; their significance and persistence in the future is under study. Abortions were more frequent and intra-uterine growth was retarded in a proportion of babies born to exposed mother. It is clear that monitoring of ill-effects and care of the afflicted have to be carried out for years.

The propensity of the Cyanates in forming reactive compounds with active groups in biological materials such as, proteins and amino-acids is well known. Of considerable significance are some of the studies being conducted which shed light on the occurrence of the phenomenon of Carbamoylation of haemoglobin amongst the exposed population in Bhopal. In the present document prepared by the ICMR, the grim story of the human effects of exposure to gas in Bhopal as it unfolded itself, based on autopsy, toxicological and experimental studies, has been presented. In addition, other clinical dimensions amongst the survivors and their sequel and future management are discussed.

Initially, in the context of legal issues, it took some time for lifting of the official embargo on publication of the scientific results. Gradually, on the lines of similar scientific publications by other agencies like, the Grant Medical College, Mumbai, CSIR, DRDE, the ICMR also published Special Supplement of some of Bhopal-related Research Projects. Subsequently, it was decided to consolidate the results of the several ICMR studies into a series of 'comprehensive compendiums' encompassing all published and unpublished technical Reports. The First Technical Report related to Epidemiological studies was published in 2007, followed by the Second Technical Report in 2008 on Clinical studies.

Incidentally brief part of Third Technical Report on Pathology & Toxicology had been submitted earlier to Dr. CR Krishnamurthy (1987), Chairman of the Bhopal Gas Commission and was incorporated in its Final Report. However, in view of the 'Historically Unique' and original scientific evidence gathered in the course of the Pathology & Toxicology Project of the ICMR, it has been decided to elaborate separately the several facets in the Third ICMR Technical Report of the BGD Series. It is proposed to present the information under different Sections, with appropriate Annexure/s to avoid repetition. The latter would contain copies of relevant published papers or a brief write-up of a topic investigated. Thereby, it is hoped to cover adequately, and without duplication, the several inter-related investigations that were undertaken.

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CHAPTER

2

AN OVERVIEW: PATHOLOGY & TOXICOLOGY

THE man-made, Bhopal Gas Disaster occurred at midnight of 2nd December, 1984. It was a cold winter night for the residents of Bhopal; when most of the people were in deep slumber. The worst tragedy recorded so far in the annals of chemical industry led to heavy mortality and morbidity. In the midst of several controversies, a large quantum of new and convincing scientific evidence was generated. Though it may sound odd, the opposition served a useful purpose by enforcing greater scientific criticality and objectivity. But not all the results could be published, due to earlier legal embargos and subsequent demise of members of the team.

But the Bhopal Gas Disaster, in general, and the Toxicological studies in particular, had inherent constraints. The lack of knowledge about the toxicity of the offending chemical, Methyl IsoCyanate, is not expected to be resolved in the throes of a Disaster! Willy-nilly partial pyrolysis of MIC was accepted to some extent, although the amount of HCN generated in the process was contested. Simply due to lack of sufficient evidence about Temperature & Pressure, changes attained within Tank 610 E, possible liberation of HCN remained a much debated issue, But the UCC objected to it on puerile grounds, such as uncertainty about the temperature attained within Tank 610 E and in the absence of scientific information about the concentration of HCN liberated at the suspected lower temperature range, viz., 200°C, 300°C and 420°C. In parenthesis, UCC itself could have advised on the basis of a simulated accident on a miniature scale in its Plants, prior to BGD or even subsequently in its Factory in Connecticut, USA (Sriramachari, 2005). In retrospect, it is also noteworthy that the UCC which initially had even indicated that 'cyanide antidotes' could be tried, had actually retracted and let loose (both in Bhopal and the world at large), a subtle campaign of misinformation, which impeded timely remedial therapeutic measures.

With years of experience in investigative and reconstructive Forensic Medicine, Dr. Heeresh Chandra, often pondered over the following scientific issues:-

1. Why was the refrigeration unit removed six months prior to the event?
2. Since when the 'cooking of the event' was going on in the Tank which was filled against all norms and kept at ambient temperature for over eight weeks,
3. What were the series of polymerisation reactions that could have taken place within the Tank?
4. What was the role of temperature and pressure during the reaction process and escape of the contents?
5. Why were the hydrants not effectively functional?
6. What could be the composition of the contents, i.e. reaction products in the gaseous or vapour form and particulate matter that were emitted from the Tank?
7. Why the scrubber was out of action and had insufficient capacity, much below the proportionate capacity of neutralisation of methyl isocyanate?
8. What constituents and what quantities people had inhaled at rest or while fleeing from their homes.
9. Why so many people died due to a commercially manufactured material and stored against all norms and never was it indicated, that it could be so harmful to human, animal and plant life.

Mystery of MIC Unfolded: Soon after the Bhopal Gas Disaster, the enigma of MIC unleashed itself. Dr. Devkumar & Dr. Mukherjee (1985) were the first in India to draw public attention to the high reactivity of MIC. It was followed by more authoritative accounts in 1985 by Dr. Varadarajan Committee Report. According to the Report, there was a massive leak of MIC, which was lying stored for a long period in the incriminated Tank 610 E of the Pesticide Plant of UCIL. The

several hypotheses attributed to this disaster, include 'prolonged bulk storage of 42 Tons of MIC, non-functioning refrigeration system, breakdown of the alarm systems for regulation of Temperature & Pressure, malfunctioning of scrubber facilities, etc. Above all, the reaction seems to have been triggered off by the sudden and massive influx of several gallons of water inadvertently, during the routine clean-up! One or more of these factors might have contributed to the accidental and uncontrolled 'runaway reaction'. From all accounts, the gas released in Bhopal is not due to just leakage of cold MIC, comparable to leakage of a single chemical like ammonia, sulphuric acid, phosgene or even hydro-cyanic acid. The presence of an array of multiple chemicals was demonstrated independently by UCC as well as NCL, Pune on behalf of CSIR. At that time, the possible toxicity of none of the compounds, including that of MIC, was known. In a later search of the Tank Residue for Toxic Nitrile Compounds, the Toxicology Project Group of ICMR also had occasion to repeat the Tank Residue Analysis, for possible presence of Nitrile Compounds. Apart from the presence of HCN at a concentration of 0.5%, there was an array of 9 or 10 additional unidentified chemical compounds, raising the total to 21. In the process, several issues related to MIC Toxicity were satisfactorily resolved beyond the original expectations. Only a few, like the quest for Toxic Nitriles & Chloro-tropism of MIC amongst the unidentified compounds in the Tank Residue, are still open questions.

The Holocaust: Indeed, in the Bhopal Gas Tragedy, the time-honoured aphorism that 'The Dead Teach the Living' seems to have emerged as a veritable truth! Within hours of the Toxic Gas Leak in the UCIL Factory, people started dying and an unprecedented flood of dead bodies were brought to the Morgue of the Medico Legal Institute (MLI), within the precincts of Mahatma Gandhi Medical College (MGMC), Bhopal. While in the first two days, literally hundreds of bodies were received, over the next three weeks, there was an abrupt decline to less than a handful '***per diem'.*** But Clinical Manifestations amongst the survivors, notably visual disturbances and severe ARDS, neuromuscular infirmities & Reproductive losses became apparent.

Pathophysiology: Distinct patterns of the Immediate, Early & Late deaths seem to suggest diverse features of toxicity, such as 'acute & severe', 'sub-acute & moderate' and 'chronic & mild' perhaps mediated by one or more of the different Toxic Chemicals generated in the run-away reaction of MIC. While several bodies showed copious frothing at the mouth and nostrils, most of the bodies, showed on opening a pinkish appearance of the skin and a Cherry Red Discolouration of the Lungs, Brain & Other Organs.

Apart from the ill-understood patho-physiological basis or mechanism, the victims, particularly severely ill survivors were affected by ARDS. Minutes after the NaTS injection, there were equally dramatic effects, accompanied by Respiratory & Neuro-muscular relief, suggestive of prompt detoxification. Thus, both the Autopsy & Clinical Pathology findings prompted Dr. Heeresh Chandra to suspect 'acute cyanide poisoning'. A couple of days later, it was endorsed by Dr. Max Daunderer, the visiting German Toxicologist, who had come fully equipped with emergency diagnostic kits and a big antidote consignment of $Na_2S_2O_3$ (believed on a conservative estimate to be at least 10,000 vials). In retrospect, he would not have done so, if there were no ***prima facie*** basis for such emergency therapeutics! Perhaps due to the raging controversy about 'cyanide toxicity issue', he was forced to go back. Fortunately, Dr. Heeresh Chandra salvaged the therapeutic approach for the ultimate good of the afflicted people. Similarly by establishing the validity of urinary SCN^- as a dependable therapeutic test, Dr. Sriramachari ensured the rationale of NaTS therapy, through the 1st DBCT onwards.

Pyrolysis of MIC; Fortuitous Evidence: Two years prior to the Bhopal Disaster, a unique & scientific publication by 'Blake and by Ijadi-Maghsoodi' entitled "Kinetics and Mechanism of Thermal Decomposition of MIC, submitted in 1981 was published in November 1982. Therein, it was convincingly demonstrated that pyrolysis of MIC at 427°C-570°C, resulted in formation of two distinct classes of chemical reactions, with separate clusters of end-products. The predominant radical chain reaction is associated with liberation of CO, H_2 and HCN through the ***major route***; and bi-molecular formation of N,N´-dimethyl carbo-di-imide and CO_2 through the ***minor route***. They also suggested and indicated the steps in the formation of such ***adducts,*** resulting in retention of the highly volatile and toxic HCN molecules within droplets of MIC in the liquid phase. They have also cited an earlier report about such reactivity of MIC & HCN published as early as 1927 by Slotta & Tshesche. There was also a similar account of Adduct formation in 1967 by Patton.

It would appear that BGD was neither manifestation of direct cyanide poisoning nor MIC alone, but was the combined affect of 'MIC~HCN Adduct' following pyrolysis. The 'Toxic Cloud' containing the adduct was inhaled by the exposed population in Bhopal. Possible ***in vivo*** dissociation of the Adduct would explain the near-fatal phenomenon

of ARDS, due to blockage of ***cytochrome c oxidase*** (a_3, a sub-unit system) of ATP cycle. Starting from the 1st DBCT, ICMR investigators have reported positive evidence of elevated serum and/or urinary SCN^- levels in pre and post NaTS-treated survivors. It may be recalled that independently, the Bombay Group (Naik et al., 1986a & 1986b; Irani & Mahashur, 1986) partially confirmed the moderate elevation of 'blood thiocyanate' levels in a large series of victims subjected to Laboratory Investigations. They attributed it to consumption of contaminated water, while therapeutic response to NaTS was not investigated. Dr. Heeresh Chandra and his team were quite successful on this front as will be demonstrated in later section of this Report, related to Issues of Cyanide Toxicity. Incidentally, the entire data was rechecked periodically, as and when new and sophisticated equipment became available to the Investigators.

By contrast, inhalation of intact MIC moieties seem to lead to widespread, but non-fatal, tissue binding typified by N- & S-Carbamoylation of end-terminal amino acids and sulphydryl groups of S Transferases. While the former is a uni-directional and irreversible process, the latter metabolically active S-Carbamoylation of Reduced Glutathione & associated Trans-Carbamoylation Phenomena can adversely affect Sulphur-Transferases like Rhodanese. The resulting blockage of the 'Triple Sulphydryl-based Catalytic Apparatus' (Heinrickson, 1983) could have contributed to recurrent or chronic cyanide toxicity or neo-cyanogenesis with a positive response to exogenous NaTS ($Na_2S_2O_3$). Incidentally, Srivastava and associates of Dr. PK Ray's team at ITRC, demonstrated sustained drop in GSH levels (Reduced Glutathione) of the Bhopal victims, although no explanation was offered at that time (Srivastava et al., 1988).

Strangely, while the paper of Blake and Ijadi-Maghsoodi virtually portends the Bhopal Gas Disaster of 1984, it has been ignored not only by UCC but also by most of the participants in the Bhopal Gas Disaster study, and with notable exceptions, even the vast majority of scientists and institutions across the world. Serendipitously, several aspects of the hypothesis of Blake and Ijadi-Maghsoodi, were adapted by the interlinked unified scientific teams at the MLI~IOP~DRDE, AIIMS~GBPH, who were involved in Pathology & Toxicology aspects of Bhopal Gas Tragedy. By taking cognisance of the above seminal article, each one of the teams was greatly benefited in their scientific pursuits of the underlying rationale of BGT Projects which practically addressed the 'Biochemical Lesion'.

New Trail of Research: For the record, a few examples of such specific interlinks are referred to in passing. Thus, Dr. Bhattacharya et al., (1987), established that pyrolysis of MIC even at a relatively lower temperature of 350°C, confirmed a release of 4.72% of HCN; one can anticipate greater release at still higher temperatures possible in the suspected Tank! Thus, the initial criticism about non-applicability of pyrolysis of MIC at 427°C-570°C is untenable in the Bhopal scenario and can be ignored. Secondly, Dr. RK Shrivastava and associates clarified a few crucial issues. It was shown that exposure of experimental animals to non-pyrolysed MIC was definitely accompanied by neo-cyanogenesis, as demonstrated by the significant elevation of serum thiocyanate levels, by the end of 1 hour, though not after 4 and 24 hours. It could be a phenomenon of one-time clearance of all the available endogenous cyanide molecules; naturally it would take time for re-accumulation of a critical mass of cyanide molecules. In parenthesis, they could have re-analysed at longer intervals when S-Carbamoylation is stabilised. On the other hand, animals exposed to pyrolysed MIC, even at a higher temperature and concentration, failed to show any increase of blood thiocyanate levels. Such a divergence can be due to extra-corporeal variations, including drift of lighter HCN in the absence of pressurised set-up as in Experiments of Blake and Ijadi-Maghsoodi.

In Quest for the Mystery of Bhopal Malady: The Toxicology Teams of ICMR veered around partial pyrolysis of MIC, followed by 'Cyanogenesis & adduct formation'. The several items listed in the *major route* viz., CO, H_2 & HCN were pursued seriously by Dr. Sriramachari & Associates in Bhopal. Apart from the intact MIC, the gaseous products seem to go first into solution in MIC itself and retained within inhaled MIC droplets to form 'unstable adducts (2MIC~HCN & MIC~HCN)'. The subsequent dissociation of the adduct molecules within the body of victims seem to actually contribute to 'acute cyanide toxicity' and consequent impaired ***cytochrome c oxidase activity***.

Concurrent CO Toxicity in Bhopal: Although accepted in principle, doubts were expressed on the lethality of HCN, generated by thermal decomposition of MIC in Bhopal. However, a similar role was not even entertained for CO, which is simultaneously generated during pyrolysis of MIC. In retrospect, it can not only explain the greater vulnerability of the people who rushed out and ran in the streets of Bhopal on the fateful day, but also the finding of CO-Hb and Meth-Hb in the Bhopal survivors, especially in the Railway Colony region by Dr. SR Kamat and associates. The focal variations in the distribution of lighter components of the toxic gases could be responsible. It also explains the severity of pulmonary damage in chronic smokers in Bhopal.

Independent of the seminal article of Blake and Ijadi-Maghsoodi, in studies on weapons-emissions, pyrolysis-based association of CO and HCN have been demonstrated in the recent classical study by the National Academy of Sciences, Washington DC (Halperin., 2008). BGD only illustrates a concentrated phenomenon of such combined toxicity.

Looking into these aspects of the event, possibly associated with inexcusable neglect of norms, one has to consider the event from several angles. With such mortality and morbidity, no clear-cut identity of the poisons was possible. Therefore, the basic tenets of Forensic Medicine, viz., **"Source, Spot and Remains"** had to be looked into minutely for all possible details. Indeed there is no irrespirable gas known so far, which could 'kill in the open space' so many people and make many more morbid.

With conditions enumerated above, we decided to trace the events from the resultants, i.e., the viscera from the dead bodies and analysis of tank residue. Herculean efforts were made. Admitting ***ab-initio*** that this is an unknown field, 'autopsy tissue' were carefully preserved at minus 70°C for future analysis. Indeed, the results indicated that the viscera contained, in addition to reaction products of methyl isocyanate, numerous unidentified organic chemicals. As anticipated, many of them were constituents of the Tank Residue as well. In an Editorial of Environmental Health Perspectives. Bucher (1987), concluded that except for presence of MIC and HCN in passing, neither the actual composition of the Tank contents, nor of the poisonous cloud actually inhaled by the people of Bhopal was known. It is because of the lack of this knowledge, that experiments can not be simulated on animals. It is still a mystery as to what reformulation and re-conjugations took place in the chemicals exposed to unknown pressure and temperature within a closed Tank.

It may be pointed out that the Pathology & Toxicology Projects and Associates of the ICMR at MLI, Bhopal, IOP & GBPH, New Delhi & DRDE, Gwalior successfully undertook an integrated series of projects related to Histopathology, Human & Experimental, Clinical Pathology & Toxicology related to Bhopal Toxic Gas (Aerosol) Tragedy. In addition to Human & Experimental Autopsies, the studies included secondary changes such as, Hb, alternate pathways of Tissue Oxygenation, through temporarily elevated 2-3 DPG pathways, a large series of Blood Cyanide estimations and massive data on Urinary SCN levels in over 18,000 individuals. Contrary to UCC's false propaganda that MIC is decomposed to MA and DMU before it crosses the alveolar-capillary barrier, it was more than established that MIC, not only reaches the blood but also several tissues and organs for at least a period of 4 months! N-Carbamoylation has been demonstrated both indirectly by the TNBS Technique, but also by the more specific N-Carbamoylation Technique for each individual Amino Acid. Due to lack of appropriate equipment, S-Carbamoylation could not be achieved, but was more than compensated by the pioneering work in 1991 by Baillie & Slatter. Initially, Bhopal casualties appear to be caused by the dual toxicity of '***MIC~HCN Adduct'***, with dramatic response to NaTS, whereas the late non-fatal sequaelae are traceable to self-limited S~N-Carbamoylation. However, the possibilities of Genetic abnormalities or mutations have to be carefully ruled-out, before concluding the Bhopal Studies.

Toxicological studies have clearly established that the Bhopal Disaster is not merely due to MIC alone, but its pyrolysed products giving rise to dual responses at each stage of the **Acute, Sub-Acute & Chronic Stages**. The initial **acute stage** seems to be the result of inhalation of '***MIC~HCN adduct'*** leading to acute cyanide poisoning associated with almost instantaneous deaths. In the **sub-Acute stage**, although, fatality was rapidly declined over the next few days, Ocular manifestations, ARDS and Neuromuscular weakness continued undiminished. The functional disturbances were persisting, characterised by anoxia, alteration in blood gases, compensatory mechanisms of 2-3 DPG and 'Recurrent & Delayed Cyanide Toxicity'. The still later **chronic stage** of the disaster, characterised by concurrent N & possibly S-Carbamoylation, favourably responded to NaTS therapy. There was no evidence of neo-cyanogenesis due to contribution of Nitrile Compounds of the Tank Residue which was also confirmed by Dr. Srivastava in his experimental studies at DRDE (Srivastava, 1990).

It is indeed fortunate that, inspite of several limitations, ICMR teams were able to resolve the mystery of several issues related to BGD. The enigma of "Cherry Red Discolouration" was ultimately traced to liberation of HCN in the course of pyrolysis of MIC in the run-away reaction in Tank 610 E of Bhopal. It has been established that a certain degree of thermolysis *initially* contributed to neo-Cyanogenesis and toxicity, which promptly responded to NaTS therapy. The dual toxicity of HCN~MIC, was demonstrated by elevated urinary SCN^- levels and several end-terminal Amino Acids of Clinical & Autopsy samples of Blood & Tissues positive for N-Carbamoylation, all of which cleared up by the end of four months. Apart from residual respiratory damage, still there is a need to rule out possible genomic involvement**.**

Investigations Undertaken in ICMR Project 08: People started dying within few hours of the ghastly 'Bhopal Gas Tragedy

/ Disaster'. Later, re-designated as 'MIC Aerosol Tragedy', Prof. Heeresh Chandra indeed planned and started the autopsy studies from the afternoon of 3rd December, 1984, with the assistance of medical & scientific staff of MLI and the Department of Pathology, MGMC, Bhopal. On the 12th December, 1984, the ICMR team from the Institute of Pathology, headed by Dr. S Sriramachari and Dr HMK Saxena, joined Prof. Heeresh Chandra and actively participated, not only in the conduct of autopsy studies but also undertook corresponding Histopathological & Toxicological investigations.

The current Compendium ICMR Project constitutes distinct Sections or Chapters, along with respective Annexures, Published or Unpublished as listed under Table of Contents. Many of the facets are obviously interlinked or had even arisen from the same determinants. While other Research Projects pertain to different disciplines of specialization, the Material & Methods, Observations, Evaluation, Correlation & Summary for each Toxicological Project are distinct. All relevant information has been brought together, to enable logical conclusions being drawn.

Certain portions, especially related to Toxicology, were presented in the Supreme Court and also the Scientific Commission on Bhopal under Dr. CR Krishnamurthy, on Pathological aspects. Subsequently, considerable portions were either presented or also published in reputed Indian and International journals, and have often been cited.

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CHAPTER

3 GROSS HUMAN AUTOPSY STUDIES

THE almost instantaneous early deaths of large numbers of people and animals following the Bhopal Gas Disaster generated several challenging issues. Although animal autopsy studies are beyond the scope of this Report, for the record it must be added that the several studies under the auspices of IVRI were not only complementary, but also had added implications about contamination of milk & meat components of 'human dietaries'. Human Autopsies were imperative for tracing before the causes of immediate and delayed or long term deaths and more importantly, timely administration of antidotes, long term therapeutic interventions and rehabilitation of Bhopal Gas Victims. At that time, it was also believed that autopsy findings and toxicological evidence might contribute towards fixation of compensation by UCIL for individual deaths as well as survivors. Accordingly, the information gathered was treated as ***sub judice*** for a considerable time.

Contributors to the Multi-Disciplinary Studies

In the fulfillment of the several tasks, Prof. Heeresh Chandra and his dedicated team of Departmental & Project Staff at the Medico Legal Institute, Bhopal, undertook a comprehensive series of Autopsy and Clinical Toxicology studies in collaboration with Prof. Darbari & Prof. Kanhere of the Dept. of Pathology of MG Medical College, Bhopal. Soon thereafter they were joined by Dr. S Sriramachari & Dr. HMK Saxena of the Institute of Pathology of ICMR, New Delhi. The Clinical Toxicological studies were undertaken in collaboration with several selected specialists like Prof. A Ramaiah of Dept. of Biochemistry of AIIMS, Prof. PS Narayanan of GB Pant Hospital and Dr. Patil of DIPAS, Brig. Lakshmipathi & Col. Sharma of INMAS, New Delhi. Last but not the least, was the abiding multi-pronged collaboration relevant with DRDE, Gwalior, through its Director, Dr. PK Ramachandran, Dr. K Jeevaratnam and their colleagues. The detailed results were submitted to the ICMR annually, for critical review and approval by the respective Advisory Groups. In the consolidated Report only the gist of the different studies are presented appropriately and in a cogent manner as Annexures under each Section are included in the Current Report or Compendium of work on Pathology & Toxicology related to Bhopal Gas Disaster under ICMR auspices.

Human Autopsy Findings

In the ultimate analysis of the Bhopal Gas Tragedy, the early deaths i.e. of December 1984 assume great importance for several reasons. Firstly, all the deaths can be traced to the exposure, inhalation and consumption of the mysterious contents of the aerosol that spread across the city of Bhopal. Secondly, it was felt that the early autopsies might provide immediate clues for the treatment of the critically ill survivors. Indeed the suspicion of 'cyanide toxicity' in the very early autopsies led to its prompt confirmation and institution of Sodium Thiosulphate (NaTS) therapy. Thirdly, automatic acceptance of such early deaths being caused by exposure to irrespirable gases and chemicals cannot be questioned legally and would act as a medico-legal precedent for purposes for claims for 'compensation' for even subsequent deaths as a consequence of the exposure. Further as Director, Medico Legal Institute, Bhopal, Prof. Heeresh Chandra also realized the need and importance of proper 'Numbering & Photographing' all the dead bodies brought to the morgue and their prompt display at key locations in Bhopal. A couple of the earlier pictures are included in the Report (Figure 3.1 a&b; 3.2 a&b), with a view to illustrate the scientific methods that were adopted even at the peak of the disaster.

The Rapidly Advancing Death Wave

Almost with in hours after the Gas Leak, from early morning of 3rd December, large numbers of people started dying and innumerable dead bodies were brought to the MLI morgue, attached to the MG Medical College. On the first day

Figure 3.1 (a & b). First Batch of Dead Bodies brought MLI and sets of identification photographs of Bhopal Gas Victims

Figure 3.2 (a&b). Photographs of Dead Animals in Bhopal Gas Disaster

Table 3.1. Postmortem Details

Date Dec 1984	3	4	5	6-10	11-15	16-20	21-25	26-30	Total
Number of Bodies Received	311	250	59	65	20	11	5	10	731
Only External Noted (%)	156 (50.2)	154 (61.6)	27 (45.7)	-	-	-	-	-	337 (46.1)
Complete Autopsy Done (%)	155 (49.8)	96 (38.4)	32 (54.3)	65 (100)	20 (100)	11 (100)	5 (100)	10 (100)	394 (53.9)

i.e., 3rd December there were as many as 311 bodies, followed by 250 on 4th and 59 on 5th (Table 3.1). Although there was a rapid decline thereafter, in a large proportion of exposees high levels of morbidity were encountered.

Even before embarking on the massive autopsy study that lay ahead, the ***pros and cons*** of performing in each case' both 'external and internal examination' were thoroughly discussed. In view of the possible risks to the limited personnel and 'working staff' of MLI, if all the dead bodies were to be opened up, it was decided to 'play safe' by restricting the 'internal examination' to a limited number of cases. Since all the deaths were due to a single episode, it was decided to carry out complete autopsies on a reasonably good proportion of cases according to the standard procedure and return the remaining bodies after recording the **'external findings'** and ensuring proper identification. Deaths recorded in the month of December 1984 were 731; out of them 620 deaths were in the first three days of the disaster. For reasons described above, **'internal examinations'** were carried out on 155 on 3rd, 96 on 4th and 32 on 5th December 1984. With reduction of numbers from 6th onwards till end of December 1984, full autopsies were performed carefully on all the bodies received **(Figure 3.3).**

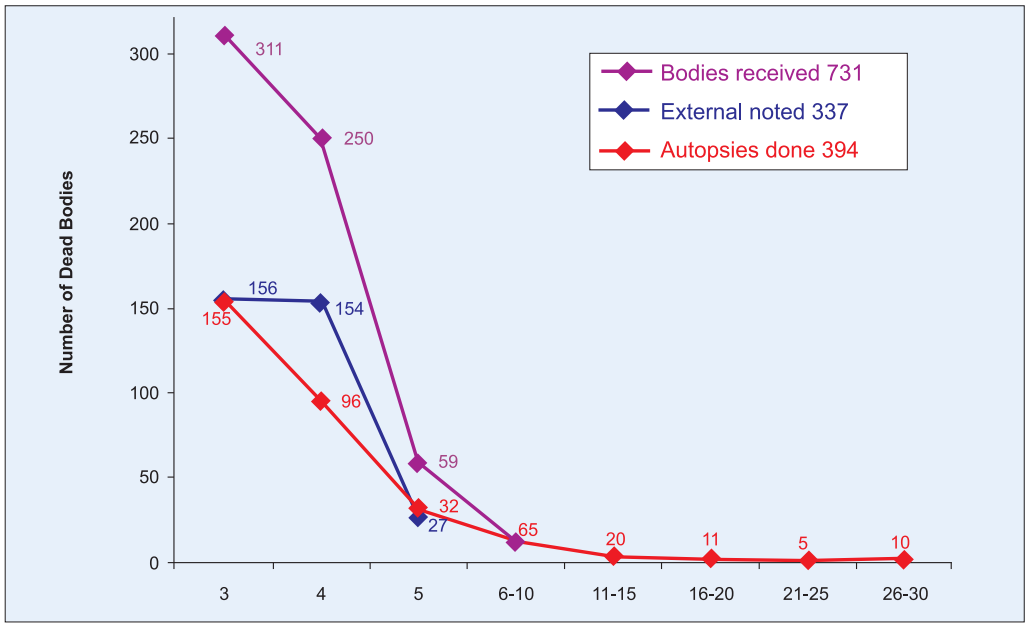


Figure 3.3. Details of Bodies Received on *Per-Diem* Basis & Autopsy Done

External Findings

These findings are summarised date-wise in the accompanying Table 3.2 with a view to enable study of comparative developments from 3rd to 30th December 1984.

i. Sex distribution: In general death amongst males was higher than in females.

ii. Age wise, out of 731 bodies received, nearly 1/3rd were of children i.e. 260 (Figure 3.4).

iii. Rigor Mortis was strong and present all over the body in 666 out of 731 cases**.** Possibly, it is due to physical fatigue generated by superimposition of lactic acid following sudden and severe hypoxia as a result of altered blood physiology confirmed during **Internal Autopsy Findings (*vide infra*)** (Figure 3.5).

iv. Hypostasis was light pink in colour and seen initially on the posterior aspect which had later extended all over. It was noteworthy that none of the bodies from the early deaths showed evidence of decomposition, although some were more than 36 hours old. On the contrary, the abdomen in some cases was "Spoon Shaped" rather than bloated.

v. In most of the bodies, the conjunctiva was congested (Figure 3.6) and the mouth was half-open and both the facies and bodies were pale. The nose and mouth had a plug of massive froth with air bubbles (Figure 3.7), and in some

Table 3.2. External Findings (+ve Cases in %)

External Findings	Dec 1984	3	4	5	6-10	11-15	16-20	21-25	26-30	Mean
Sex	Male	60.1	37.6	47.4	61.5	70	54.5	80	70	58.8
	Female	39.8	42.4	32.5	38.4	30	45.5	20	30	41.2
Eye Conjunctiva (congested)		84.2	98.8	98.3	89.2	90	63.6	60	10	89.4
Absence of Cyanosis		94.8	98.4	100	100	100	90	60	10	95.6
Skin Colour	Pink	60.7	50.4	40.6	64.6	60	81.8	40	30	91.1
	Pink & Dry	15.7	36.8	32.2	12.3	5	-	20	10	0.2
	Dry	3.5	0.8	-	1.5	5	9	-	-	7.9
	Yellow	0.35	-	-	-	-	-	-	-	0.1
Rigor Mortis	Full	99	77.6	98.3	98.4	95	81.8	100	90	91.1
	Rising	0.3	-	1.6	-	-	-	-	-	0.2
	Passing	-	22.8	-	-	-	-	-	10	7.9
Froth	Only Nose	31.5	31.6	6.7	32.3	10	9	-	-	28
	Only Mouth	1.0	6.4	1.6	4.6	5	27.2	-	10	3.8
	Nose & Mouth	51.1	58.4	89.8	56.9	3	27.2	20	10	55.5
Neck Veins (engorged)		77.1	87.2	74.5	52.3	25	27.2	20	10	74.6

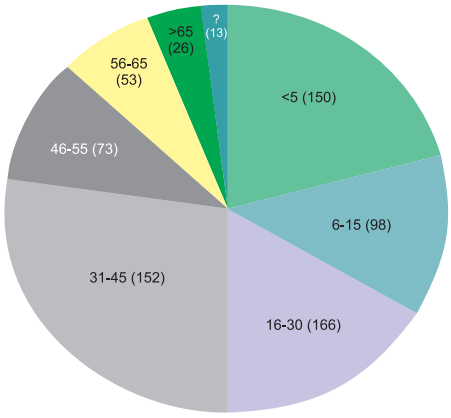


Figure 3.4. Age-wise distribution of Postmortems (Total 731 Cases)

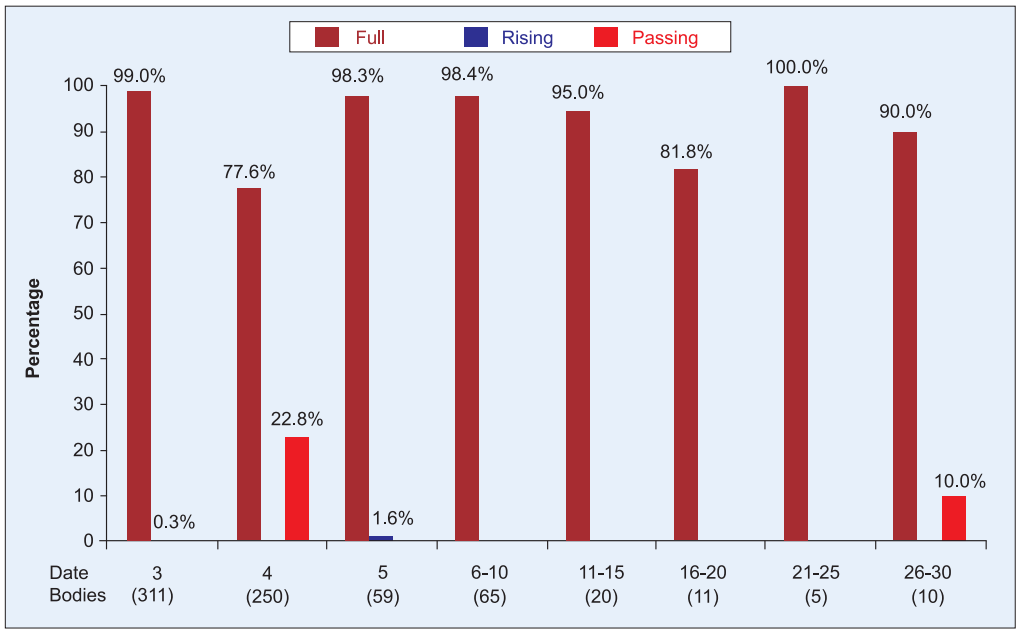


Figure 3.5. Rigor Mortis

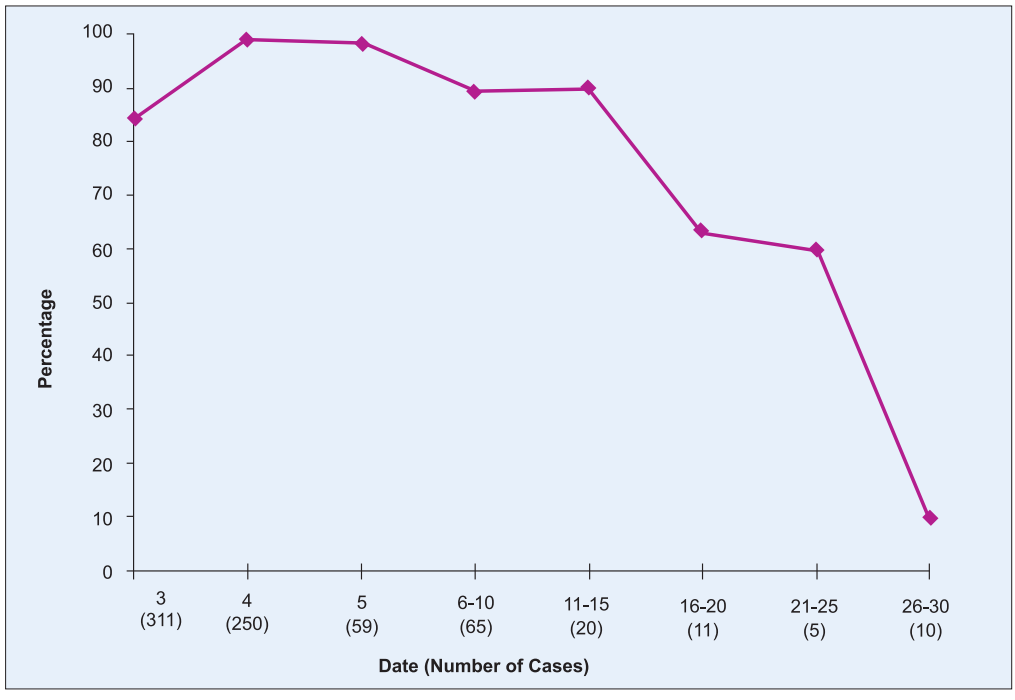
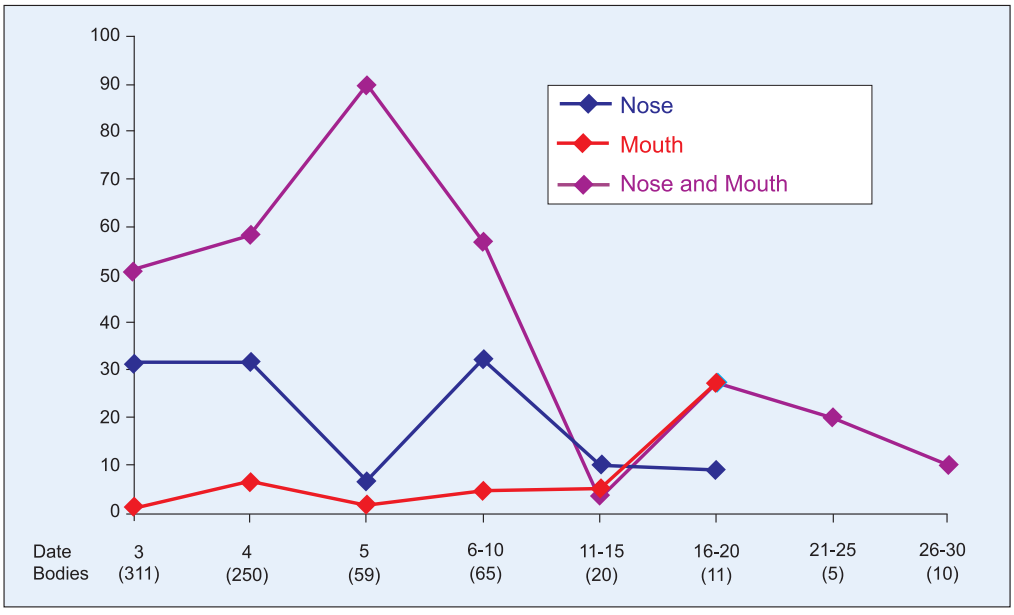
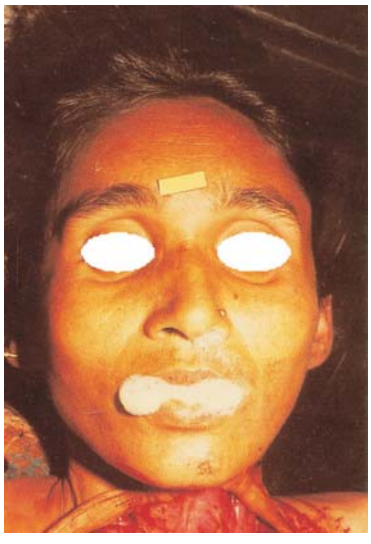


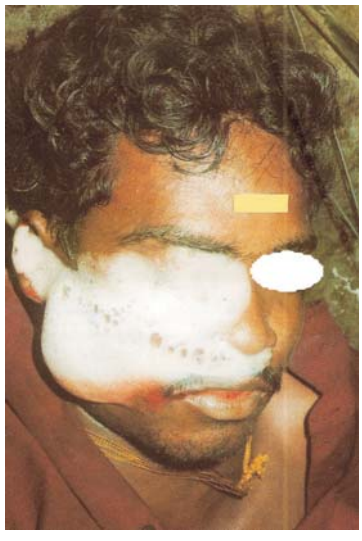
Figure 3.6. Eye Conjunctiva (congested)

cases very light yellow to clear fluid was oozing with no blood tinge. On tilting the body, clear fluid was coming out from the mouth and nostrils like a tap. Jugular veins were prominent in a large proportion of cases. Practically there was no evidence of cyanosis in the bodies brought until 20th of Dec 1984.





ORAL FROTHING



NASAL FROTHING

Figure 3.7. Frothing – Nasal & Oral

vi. It was noticed that the usual post-mortem lividity or cyanosis was not present (Figure 3.8); instead, there was a pinkish discolouration over all parts of the body.

INTERNAL FINDINGS

It was noteworthy that on opening the bodies a typical characteristic odour was perceived. This was smelt in about 70% dead bodies on 3rd and all the dead bodies on 4th; afterwards the smell gradually decreased till 20th (Table 3.3).

Table 3.3. Internal Findings: Odour on the opening of the Body (+ve Cases in %)

Date	Dec 1984	3	4	5	6-10	11-15	16-20	21-25	26-30	Total
Number of Complete Autopsies		155	96	32	65	20	11	5	10	394
Odour - Typical (%)		70.9	100	68.7	27.6	15	5	-	-	63.4

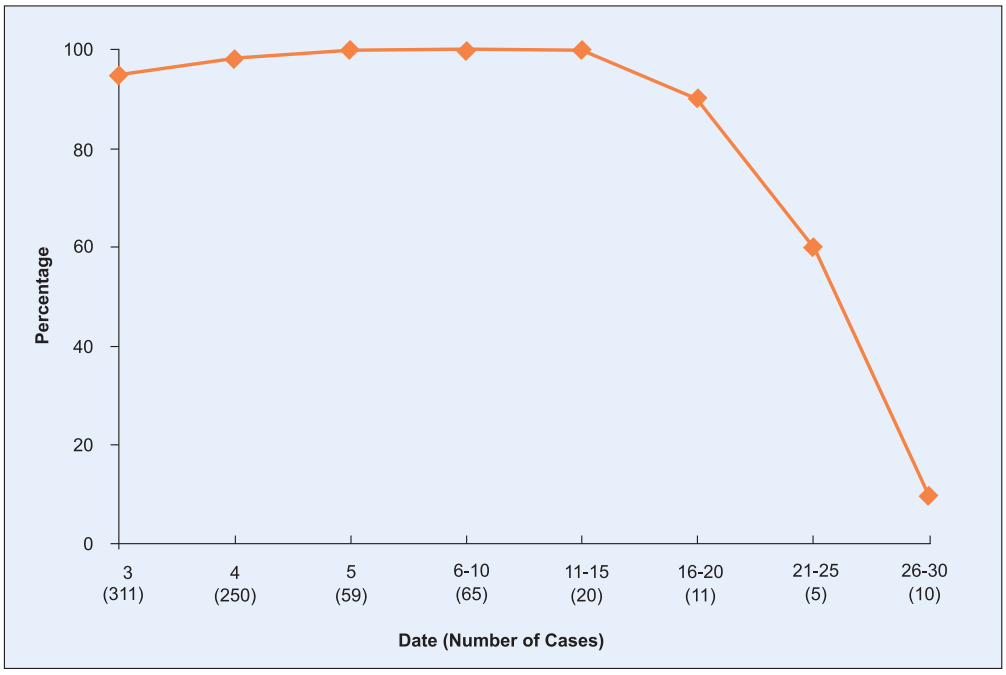


Figure 3.8. Cyanosis not detected

Blood

Another important feature from the earliest autopsies in Bhopal was marked congestion and '**characteristic cherry-red colour'** of the entire viscera and especially the lungs, which aroused suspicion of **'cyanide toxicity'** by Prof. Heeresh Chandra. The hypothesis was successfully confirmed on 8th December following the serendipitous visit of Dr. Max Daunderer, a visiting German toxicologist from Munich. With the help of a Drager Tube he demonstrated successfully a cyanide level of about 2 ppm in the blood of dead victims (Hankinson, 1986). Until the end of the December the dead bodies showed a pronounced **Cherry Red Colour** of the blood**,** and some a pink colour thereafter (Figure 3.9).

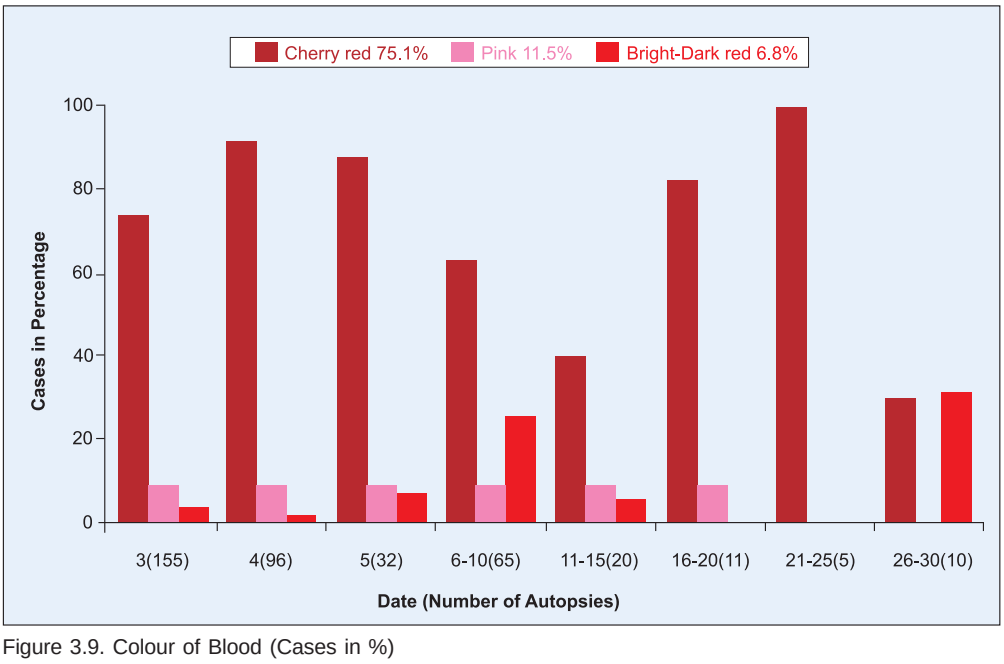


Figure 3.9. Colour of Blood (Cases in %)

Most of the dead bodies showed overall congestion and petechial hemorrhages in the vital organs. Liver, Spleen, Brain and Kidneys showed unusual softness. Some of the cases of 1985 also showed similar findings. Generalized hemorrhage was found in many organs with altered naked eye appearance of blood. The 'white matter' was unduly

softened with ventricles full of clotted or coagulated blood. A detailed observation is appended date wise and organ wise to diagnose the episode more clearly. Dark thin fluid blood when spread over tiles appeared to be 'cherry red' in colour and aggregated into clumps as seen in agglutination. The venous side was engorged, probably indicative of acidosis. The large blood vessel contained clots in two cases and also admixed with dark purple fluid blood in one of the case.

Brain

As per the details shown in Table 3.4, from 3rd December onwards the brain was found to be severely edematous, congested and dotted with fine hemorrhagic spots on section of the cerebral hemispheres, mid brain and cerebellum. Sub-arachnoid congestion was marked with bright pink colour. Softening of white matter was also observed. Similar appearances were seen even upto the 10th of December. Later, although it started decreasing, congestion of the vessels and slight sub-arachnoid hemorrhage on cortical surfaces remained. On section some of the brains showed petechial hemorrhage in the cortical portion of the brain. There was generalized softening. Later on the brain continued to be edematous and hyperemic, especially the choroid plexuses, accompanied by cerebral softening. In all the cases, dura was thickened and congested. Brain edema was mild to moderate except in a rare case with hydrocephalus. The ventricles in these two cases were dilated and white matter i.e., cortex of the brain was reduced in thickness to about 1 cm. The walls of the ventricle were softened and necrotic; with white yellow patchy material sticking. In one case, nuclei were completely sloughed out and 3rd and 4th ventricles were full of pus (Figure 3.10; 3.11).

The generalized softness of the white matter of the brain with engorgement/ hemorrhage was most prominent feature. The blood vessels had uniform red / cherry red colour indistinguishable in arteries and veins.

Table 3.4. Internal Findings: Brain (+ve Cases in %)

Date	Dec 1984	3	4	5	6-10	11-15	16-20	21-25	26-30	Total
Number of Complete Autopsies		155	96	32	65	20	11	5	10	394
Meninges – congested		94.8	100	96.8	98.4	100	72.7	80	90	96.4
Oedema		98	100	100	98.4	100	90.9	100	100	98.7
Hemorrhage	Intra-cerebral	10.3	12.5	9.3	3	10	18.1	-	-	9.3
	Sub-arachnoid	3.8	3.1	3.1	6.1	-	-	20	10	4
	Petechial	31.6	22.9	50	72.3	80	18.1	-	30	39.3
	Petechial Intra-cerebral	1.9	5.2	15.6	4.5	10	27.2	-	10	5.5

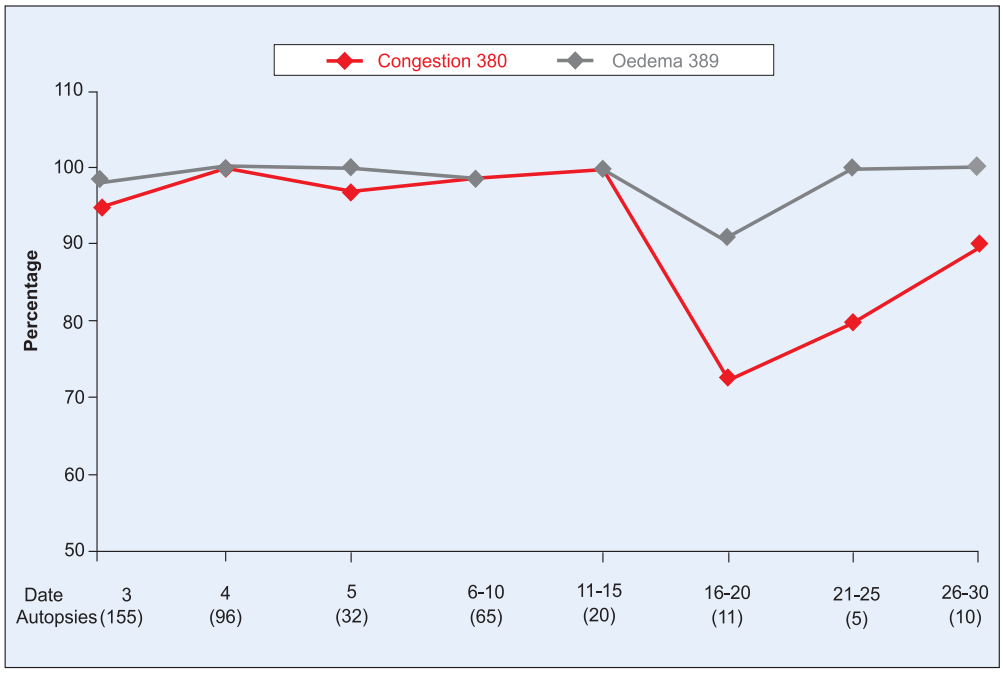


Figure 3.10. Brain Congestion and Oedema

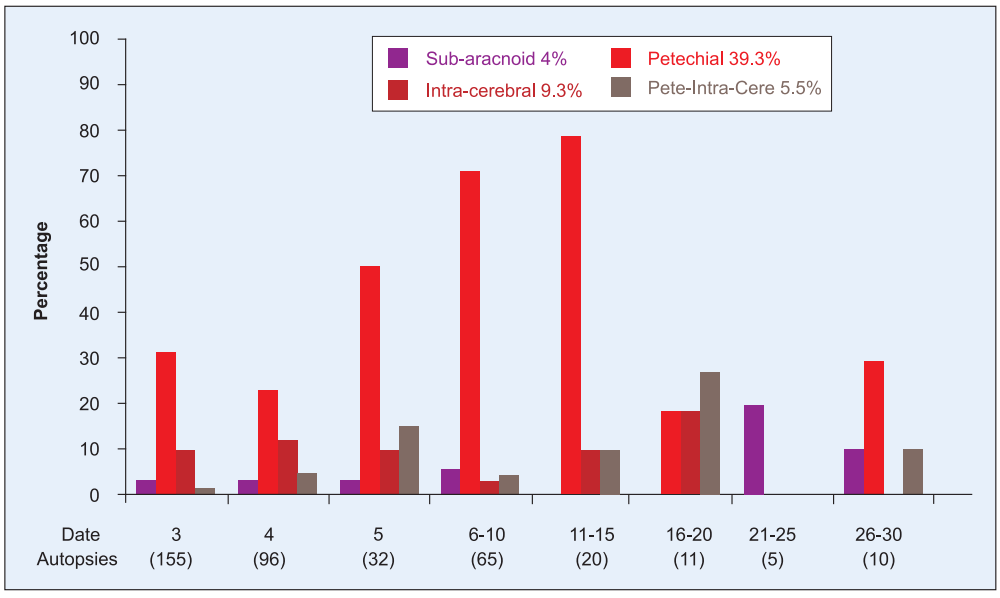


Figure 3.11. Brain Hemorrhage

Lungs

As per the details shown in Table 3.5, both the lungs were voluminous, pinkish-gray in colour, coveed by the anterior chest wall. Both thoracic cavities contained about 100 - 200 ml fluid on each side. Petechial hemorrhagic spots were present all over the lung. On section, frothy, dark pink fluid was oozing out. Usually, both lungs were severely edematous and mottled. They weighed 2 to 3 times more than the average lungs. They were soft and spongy, giving an appearance of drowning in their own secretions. Trachea contained froth and the congested mucosa appeared pink to bright red in colour. In one case serous fluid was present in the thoracic cavities, and in another the pleura was adherent to the chest wall. The visceral pleura had generalized petechial hemorrhages and cut sections of the lungs also showed small jelly-like patches of consolidation. In the following week the patches appeared ecchymosed, bigger in size and

Table 3.5. Internal Findings: Gross Pathology of Lungs (+ve Cases in %)

Date	Dec 1984	3	4	5	6-10	11-15	16-20	21-25	26-30	Total
Number of Complete Autopsies		155	96	32	65	20	11	5	10	394
Voluminous		96.7	100	100	98.4	100	90.9	100	90	97.9
Oedematous		100	100	100	98.4	100	90.9	100	90	99.2
Congested		27.2	90.6	100	96.9	100	90.9	60	90	67.7
Petechial Haemorrhage		27	21.8	18.7	49.2	30	36.3	60	40	29.9
Mottling	Gray	31.6	34.3	34.3	47.6	55	36.3	20	50	36.8
	Pink	6.5	13.5	12.5	13.8	10	9	20	10	9.6
	Gray & Pink	31.9	-	-	-	5	-	-	-	1
Bronchitis		3.8	6.2	9.3	20	25	9	20	20	9.3
Emphysematous Bullae		2.5	1.4	3.1	1.5	-	-	-	-	2.5
Cavitation		0.6	-	-	1.5	-	-	-	-	0.5
Broncho – pneumonia		0.6	11.4	21.8	32.3	15	9.0	-	-	11.1
Consolidation		0.6	4.1	3.1	35.3	70	81.8	40	70	15.4
Fibrosis		0.6	1	-	3	5	-	-	-	1
Ceasation		-	-	-	3	-	-	-	-	0.5
Lung abscess		-	-	-	1.5	-	-	-	20	0.76
Pleura	Serous	91.6	94.7	96.8	78.4	25	-	20	10	81.7
	Adherent to lungs	3.2	4.1	3.1	7.6	15	36.3	40	20	6.5
Pleural effusion		91.6	97.9	96.8	83	40	-	20	10	86.8
Pulmonary Tuberculosis		-	-	-	3	-	-	-	-	0.5

congested. The bronchioles were full with yellowish secretions. On section, frothy pink thick blood oozed out. The lungs appeared somewhat shrunken. Later on, the apical regions were found to be emphysematous with bulla on the apical surface and which collapsed on section. In the later half of December, lung weights were nearly at par with Normal; the surfaces were moist and slightly pink with a few dark patches. Posterior regions of lungs were consolidated, chocolate colored and with pitting effects. Rest of the area was congested with very few patches in the periphery showing normal lung tissue with pink colour. On section necrotic changes were present and pink colored frothy fluid oozed out (Figure 3.12; 3.13; 3.14).

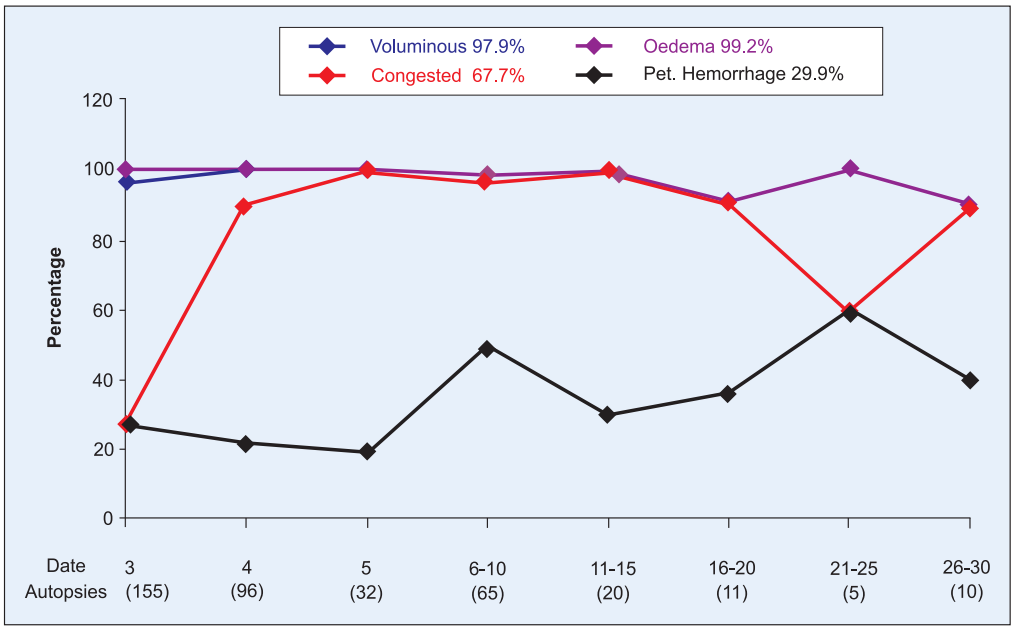


Figure 3.12. Gross Pathology of Whole Lung

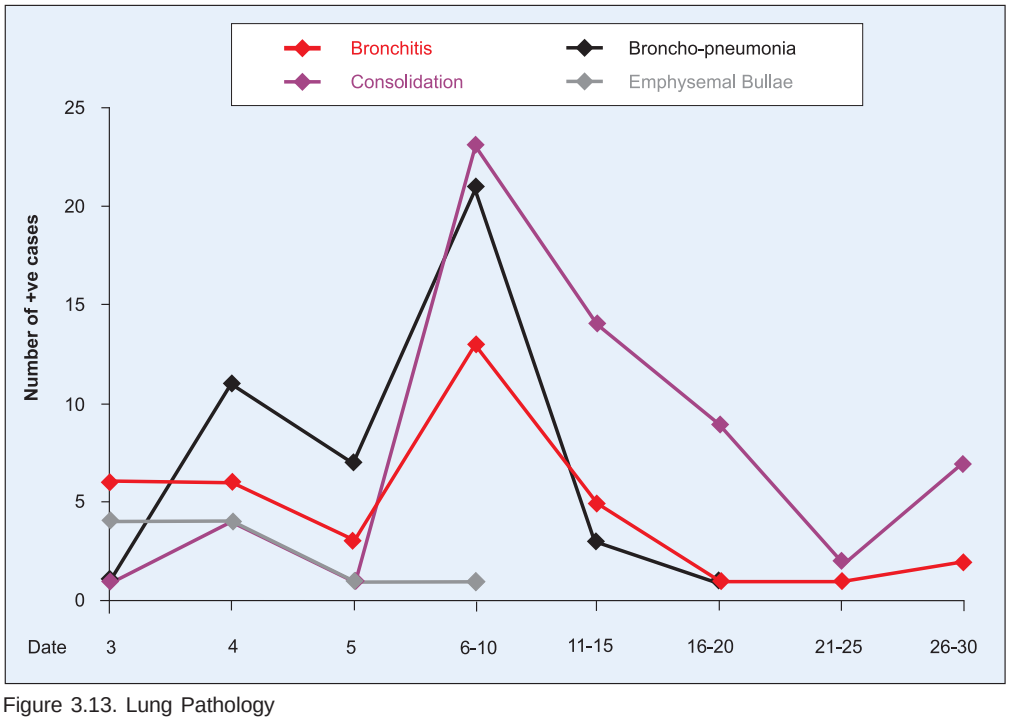


Figure 3.13. Lung Pathology

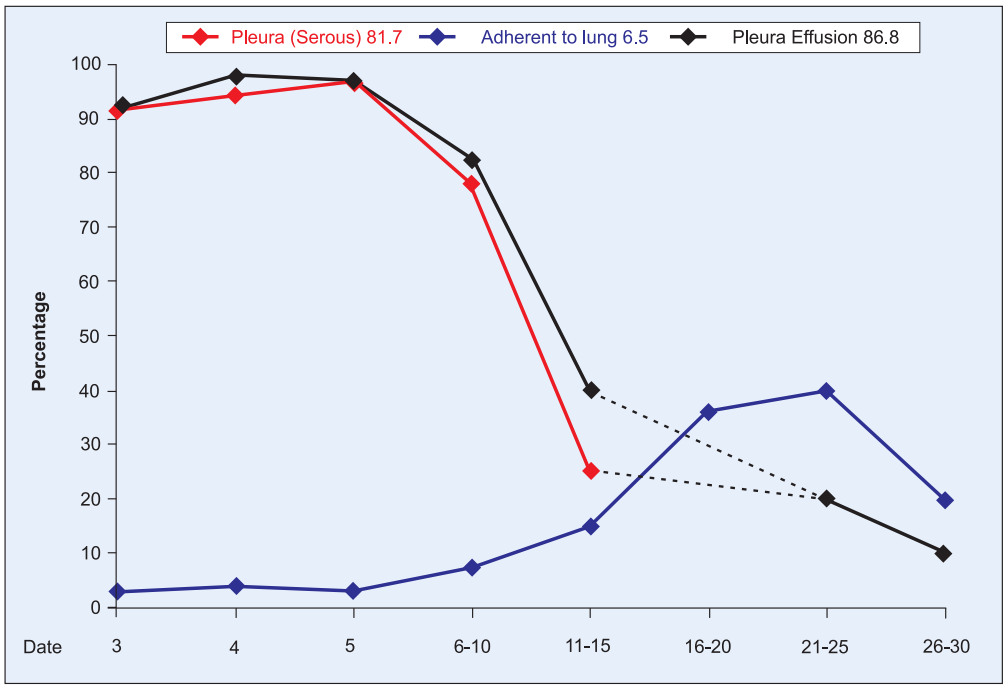


Figure 3.14. Lung Pleura

Heart

As per the details shown in Table 3.6 in the initial autopsies the heart showed clotted blood and fluid in both sides. All over the anteior and posterior surfaces there were multiple fine hemorrhages. Coronary vessels were mostly patent. The myocardium appeared to be ecchymosed and dark but in some it was pink or pale. The whole heart showed flabby appearance to touch. The visceral pericardium showed petechial hemorrhage, mostly over the left ventricle. The right side contained clotted blood while it was less on the left side. Major part of the clot was fibrinous. Recent myocardial infarction with yellow patches was seen in three cases indicating the episode at least more than 24 hours.

Table 3.6. Internal Findings: Gross Pathology of Heart (+ve Cases in %)

Date	Dec 1984	3	4	5	6-10	11-15	16-20	21-25	26-30	Total
Number of Complete Autopsies		155	96	32	65	20	11	5	10	394
Petechial Haemorrhage		47	69.7	78.1	61.5	70	63.6	40	-	57.8
Ventricular dilatation		0.6	5.2	9.3	20	15	36.6	-	40	8.3
Heart Chamber - Right	Fluid Body	40	48.9	9.3	3	-	18.1	-	-	29.4
	Clotted Blood	55.4	48.9	87.5	75.3	100	72.7	80	80	63.4
	Slight Amount With Clotted Blood	0.6	-	3.1	4.6	-	-	20	20	2
Heart Chamber - Left	Empty	32.9	42.7	46.8	27.6	45	9	40	30	33.5
	Clotted Blood	5.8	2	-	15.3	10	45.4	40	20	7.8
	Slight Amount With Clotted Blood	36.1	50	53.1	27.6	40	26.2	-	20	38.5

Stomach

Stomach was generally empty with mucosa showing mild diffused congestion. In some cases mucosa was intensely reddish with an 'hour glass' appearance, indicating levels of the resting fluid. In some cases the stomach had hypermic, congested mucosa with some haemorrhages and contained some fluid.

Intestines

Small intestine was generally found empty but in large intestine faecal matter was present. There was no distension due to absence of gases and no smell on opening, indicating defloration of G.I. bacteria.

Liver

It was generally congested, chocolate colored, flabby, soft, shrunken with icteric cut surface oozing blood. Visceral surface had petechial hemorrhages. Gall bladders were unusually distended.

Spleen

The spleen was markedly congested, brownish black in colour with generalised petechial hemorrhages in the visceral layer with pink thick blood oozing out of the cut surface.

Kidneys

Both kidneys were severely congested and dark brown in colour.

Comparative Autopsy Findings of 1985 to 1987

Brain

The overall picture in relation to brain weight did not show any marked increase in both the sexes with the subsequent passage of time after 1984. Initial haemorrhagic condition continued till 1987. Some cases of 1985 showed mild congestion in meninges, and later on significantly decreased in the following years (Table 3.7; 3.8; Figure 3.15; 3.16).

Table 3.7. December 1984

Internal Findings (Dec 84)	3	4	5	6 to 10	11 to 15	16 to 20	21 to 25	26 to 30	Total
Autopsy Done (%)	**155 (49.8)**	**96(38.4)**	**32 (54.3)**	**65 (100)**	**20 (100)**	**11 (100)**	**5 (100)**	**10 (100)**	**394 (53.9)**
Pleural Effusion	96	94	98	80	45	-	40	-	85
Bronchitis / Bronchiolitis	5	1	6	9	25	-	-	20	6
Pulmonary Oedema	100	94	100	86	100	81	100	100	96
Pulmonary Hemorrhage	24	9	12	60	30	27	60	40	26
Pneumonic Consolidation	2	1	-	26	20	81	60	60	11
Pulmonary Tuberculosis	-	-	-	1	-	-	-	20	1.5
Pleural Calcification	-	-	-	-	-	-	-	10	0.3
Sub-arachnoid Hemorrhage	4	1	3	8	-	-	20	20	4.3
Cerebral Hemorrhage	26	39	68	63	75	63	20	60	171
Cerebral Atherosclerosis	1	9	3	12	40	18	20	-	7

Table 3.8. 1985 (January to December)

Internal Findings (1985) Autopsy Done (%)	Jan-Mar 34 (100)	Apr-June 23 (100)	July-Sep 32 (100)	Oct-Dec 16 (100)	Total % 105 (100)
Pleural Effusion	-	-	-	-	-
Bronchitis / Bronchiolitis	8	8	18	18	13
Pulmonary Oedema	41	4	13	31	22
Pulmonary Haemorrhage	26	8	21	18	20
Pneumonic Consolidation	32	17	25	31	26
Pulmonary Tuberculosis	11	8	-	12	7
Pleural Calcification	-	4	13	18	7
Sub-arachnoid Haemorrhage	11	-	-	6	4
Cerebral Heamorrhage	26	8	13	6	15
Cerebral Atherosclerosis	3	12	13	6	8

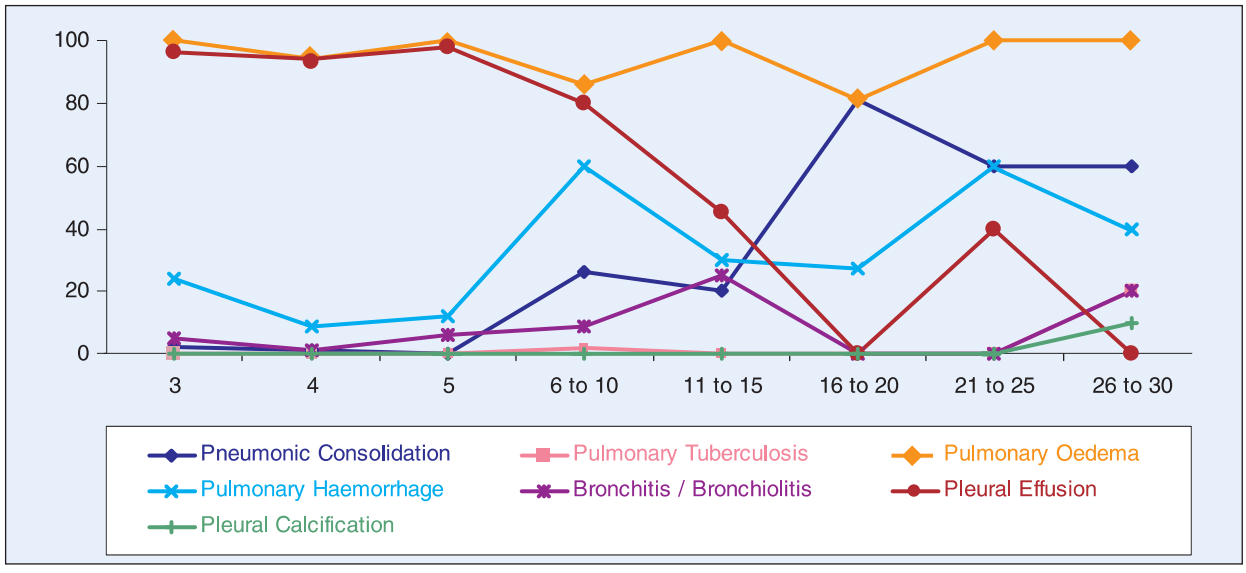


Figure 3.15

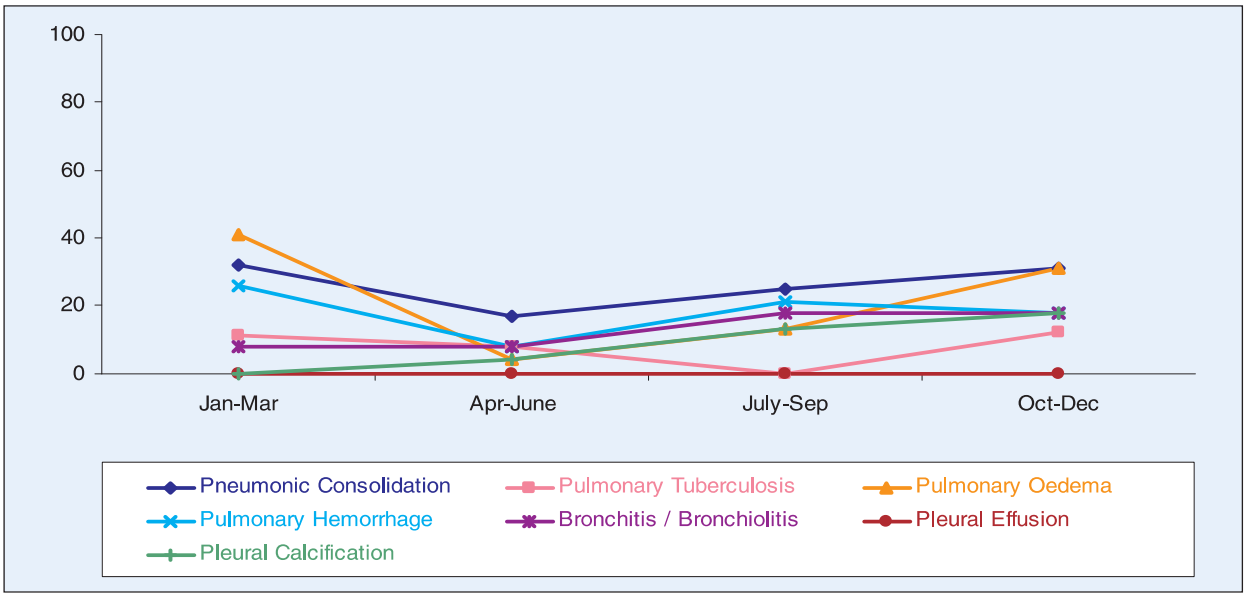


Figure 3.16

Lungs

As observed in 1984 cases, lungs weights were above normal in both the sexes. This continued till first quarter of 1985, when it became normal. But after the third quarter of 1985 till 1987 in most of the cases it was above normal. Pulmonary edema, congestion and hemorrhage were the prominent findings of 1984, also observed in less number of cases of 1985 and these findings were constantly seen in 1986 as well as 1987 cases. Pleural thickening was a noted feature in 1985 cases. Bronchitis and pulmonary consolidation was present in higher number of cases of 1985 as compared to 1984 but in the subsequent years these were present in few cases only (Table 3.7; 3.8; Figure 3.17; 3.18).

Heart

A marked increase in heart weight was observed in both the sexes. Especially from 11th December 1984 till the end of the month. After July 1987there were a significant increase in heart weight in most of the cases.

Liver

Liver constantly showed above normal values of weight in 1984 cases. But since 1985 it was within the normal

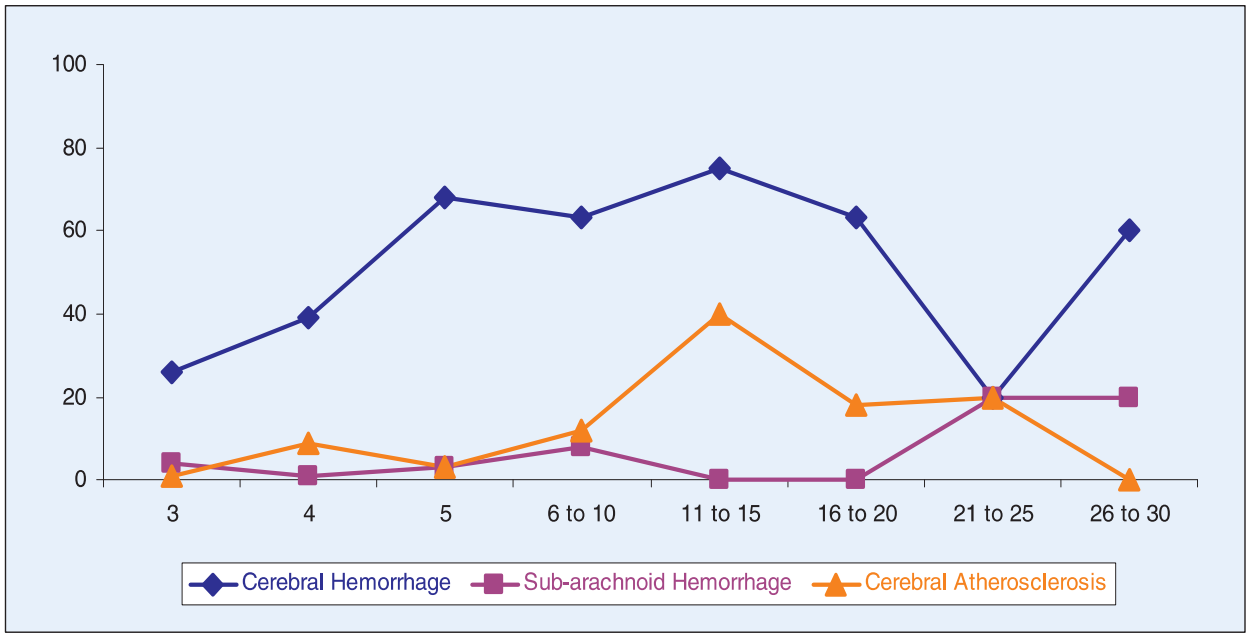


Figure 3.17

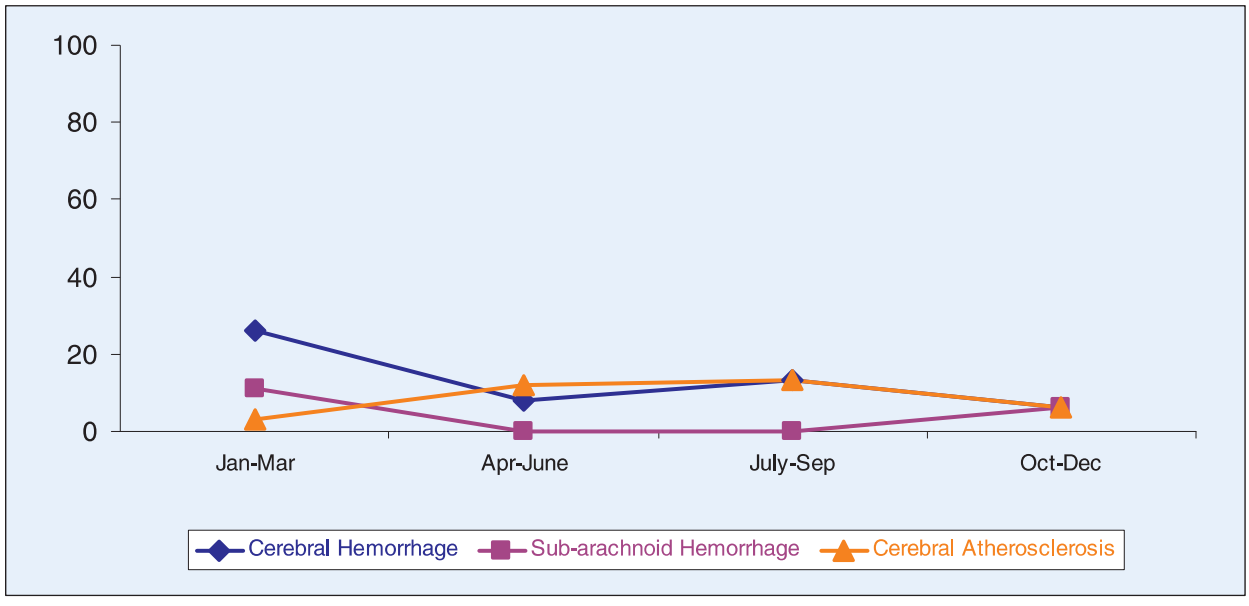


Figure 3.18

range in both the sexes. Congestion was a prominent feature of 1984 cases which was also continued in most of the cases of 1985, 86 and 87. Liver consistency was soft in most of the cases throughout this time period.

Spleen

In the initial phase of 1984 spleen showed nearly normal values of weight but since 16th December 1984, in males there was a rapid increase in weight observed till the end of month and it became normal during mid of 1985. Congestion and softening were observed in some cases.

Kidneys

Kidneys showed constantly much variation in weight, it was more prominent in females and always above normal values with slight increase during December 1984. Congestion and softening continued till 1987 in most of the cases.

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❑

CHAPTER

4 HISTOPATHOLOGICAL & EXPERIMENTAL STUDIES

Introduction

Prof. Heeresh Chandra and his colleagues started performing autopsies within 72 hours of the disaster. The entire lot of 394 autopsies on Bhopal Gas Victims were carried out at the Medico Legal Institute. The presence of a thick tenacious foamy froth covering the nose and the mouth, as illustrated in the previous Chapter, was a common post-mortem finding in the early autopsies, Likewise, the reddish discoloration of the conjunctiva was yet another common feature. Post-mortem hypostasis was not confined to the dependent parts, but was present all over the body. The lungs were enlarged and highly edematous and showed congestion, haemorrhage and consolidation. The pulmonary vessels were filled with thick viscid dark Cherry Red Blood. The bronchi and trachea were reddish in appearance and filled with white tenacious material. The heart often contained blood clot which was cherry red and some chicken-fat like material. The liver showed hemorrhagic spots. The gall bladder was distended. Frequently, the stomach and intestines showed hemorrhages in the wall and in the sub-mucosa. The spleen was shrunken and softened. The kidneys showed focal hemorrhage. The brain was 'Cherry Red' in colour. It was edematous and soft in consistency. On section, there were foci of haemorrhage, particularly in the white matter. In a few cases, there was sub-arachnoid, intra-ventricular and intra-cerebral haemorrhage.

Histopathological Studies

The Histopathological Studies were undertaken jointly with two groups of Pathologists. The 1st one comprised of Prof. NS Darbari and Colleagues of the Department of Pathology, MG Medical College, Bhopal. Although, there were very little differences with respect to the initial findings by both the Groups and by way of independent confirmation of the observations and for purpose of 'record', a brief abstract of the salient findings of the 1st study is included below, followed by a more detailed account of the 2nd Group. .

The First Histopathological Study

The study is based on a total of 40 autopsies of MLI series of victims of both sexes and all age groups gathered on a weekly basis, largely spread over the first five weeks and occasionally upto four months.

Autopsy examination of two cases that died in the first 72 hours, showed that the brunt of the attack was on the lungs, with severe necrotizing lesions affecting the bronchioles, alveoli and capillaries, along with profuse inflow of fluid into the alveoli. Necrotizing lesions were also found on the lining of the upper respiratory tract. There was very little evidence of secondary infection and cellular response in the lung was manifest as poly-morphonuclear infiltration and proliferation of alveolar macrophages

Lungs: In all cases affected lungs were heavy, voluminous, congested, edematous and more marked in the lower lobes. Histologically, the initial nine cases during the first week showed 'necrosis and desquamation of the respiratory channels', associated 'interstitial and hemorrhagic changes' accompanied by massive 'alveolar edema'; In the second week, the predominant picture in 12 out of 15 autopsies was one of 'interstitial pneumonitis' and 'bronchiolitis obliterans' filled with desquamated epithelial debris. In the following week, while there was decrease of edema, there was an increase of the inflammatory reaction. By the fifth week, alongside alveolar thickening and compensatory emphysema, interstitial fibrosis and bronchopneumonia became predominant.

Other Organs: The brain showed occasionally petechial hemorrhages over the entire period, peri-cellular and peri-capillary edema and dilatation of Virchow-Robin spaces, neuronal damage and degeneration of cerebellar Purkinje cells. In

18 cases, the heart showed evidence of interstitial edema. The liver showed foci of hepatocellular necrosis and fatty change in 15 out of the 40 cases. Infrequently, stomach and intestines showed superficial ulceration and renal tubular necrosis.

The Second Histopathological Study

The "Early as well as Long term Histopathological Studies", based on Light & Electron Microscopy was more detailed and comprehensive in the 2nd study. The ICMR team consisting of Dr. S Sriramachari, Dr. HMK Saxena, Dr. Ashok Mukherjee & their associates at Institute of Pathology, Safdarjang Hospital, New Delhi, helped the Bhopal Toxicology team from December 13th - 21st, 1984. The following description is based on a detailed report on 22 consecutive autopsies performed during this period.

"On opening the thoracic or abdominal cavities, the viscera rapidly acquired a reddish tinge. This was presumed to be due to their interaction with atmospheric oxygen. This feature was observed in the lungs, small intestines and brain. The cherry-red colour of the post-mortem specimens of various organs gave rise to a strong suspicion of cyanide poisoning (vide Annual Report, 1984 of Institute of Pathology-ICMR). The following are some of the important histopathological changes observed under Light and Electron Microscopy in the second autopsy series of 22 cases. The autopsies studied upto December 22, 1984, were subjected to detailed histopathological examination at the Institute of Pathology. In all cases, paraffin sections were stained by Haematoxylin & Eosin and where necessary for Reticulin (Gomori) or Collagen (Masson) Elastic tissue (Verhoeff) for Hyaline membranes and phagocytic debris (PAS), fat (Sudan IV), and Nissl staining procedures.

Early Histopathological Findings: Significant and consistent histopathological changes were seen in the respiratory system, characteristic of bronchitis, bronchiolitis, tracheitis and extensive and widespread edema of the lungs and extensive hemorrhages. There was total lung involvement and the parenchyma was abnormal; the lesions were however free from bacteria. By the 2nd Week of December**,** interstitial pneumonitis with ulcerative and inflammatory lesions of bronchioles was predominant. Denudation of epithelium and acute inflammatory cells were seen on the tracheal wall. Exudates consisting of neutrophils and lymphocytes and desquamated epithelial cells filled the bronchioles. By the 3rd week, the alveoli were found to be thickened and the alveolar lumen was filled with necrotic debris and macrophages with chronic inflammatory cells. The bronchioles showed necrotic epithelial debris embedded in mucin.

On 21st December, 1984, the respiratory tract and lungs showed more or less a similar pattern which was observed in the first or second week of December, 1984, by Dr. Heeresh Chandra, except for a slight reduction in the lung size. The trachea was purplish red in colour and the smaller bronchi were filled with a frothy fluid. The lungs were water-logged and appeared reddish in colour and there was copious fluid exudation from cut surfaces. Upto the end of the third week, the respiratory systems showed a consistently uniform pattern and on gross examination variable involvement of other organs was also noticed.

The pathological findings were summarized, all the important microscopic lesions were photographed in colour and the Report was made available to Prof. Heeresh Chandra. The same had also been presented at the initial meetings held in Bhopal from January 1985 onwards, submitted to the Bhopal Gas Commission under Dr. CR Krishnamurthy and has also been published in its 'Official Report' which was released in July 1987.

A summary of the pathological findings in the Lung and other Viscera is presented in Figure 4.1 to illustrate the over-all distribution of the pathological lesions observed in the series of cases in the 2nd study.

By contrast, quite often pathological changes were seen in the Brain, including intra-cerebral haemorrhage, peri-cellular and peri-capillary edema with neuronal degeneration.

Antecedent findings of tuberculosis in the lungs and GI tracts were not unexpected. Occasionally ulcers were seen in the stomach and small intestine. The liver presented features of widening of sinusoids, dissociation of hepatic cords, focal necrosis, and congestion, presence of lipofuchsin granules in hepatocytes and mononuclear infiltration of portal tracts Liver showed varying degrees of acute agonal changes like swelling, vacuolation and fatty degeneration. A substantial number showed marked enlargement of the Gall Bladder and accumulation of bile. Spleen was congested. Kidneys showed interstitial edema. The kidney showed in 3 cases, acute tubular necrosis, particularly in the proximal convoluted tubules. The renal medulla showed congestion and filling up of the collecting ducts with tubular casts (Figure 4.2).

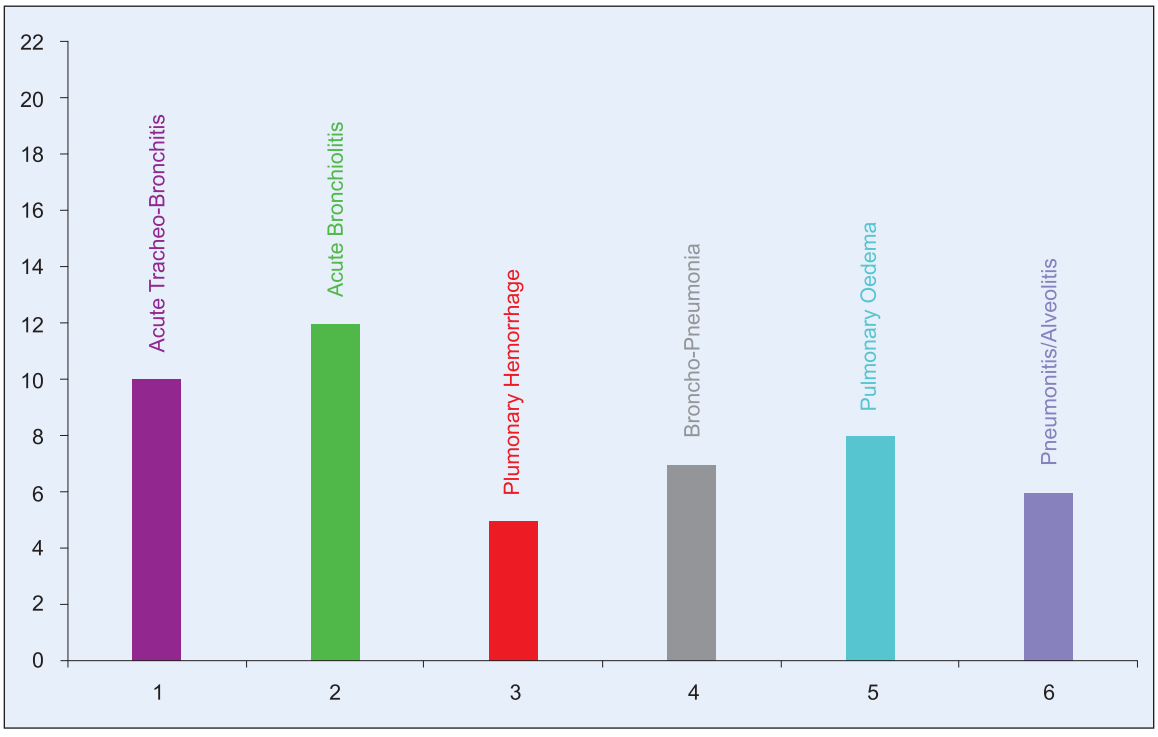

Figure 4.1. Series II: Overall Prevalence of Pulmonary Lesions

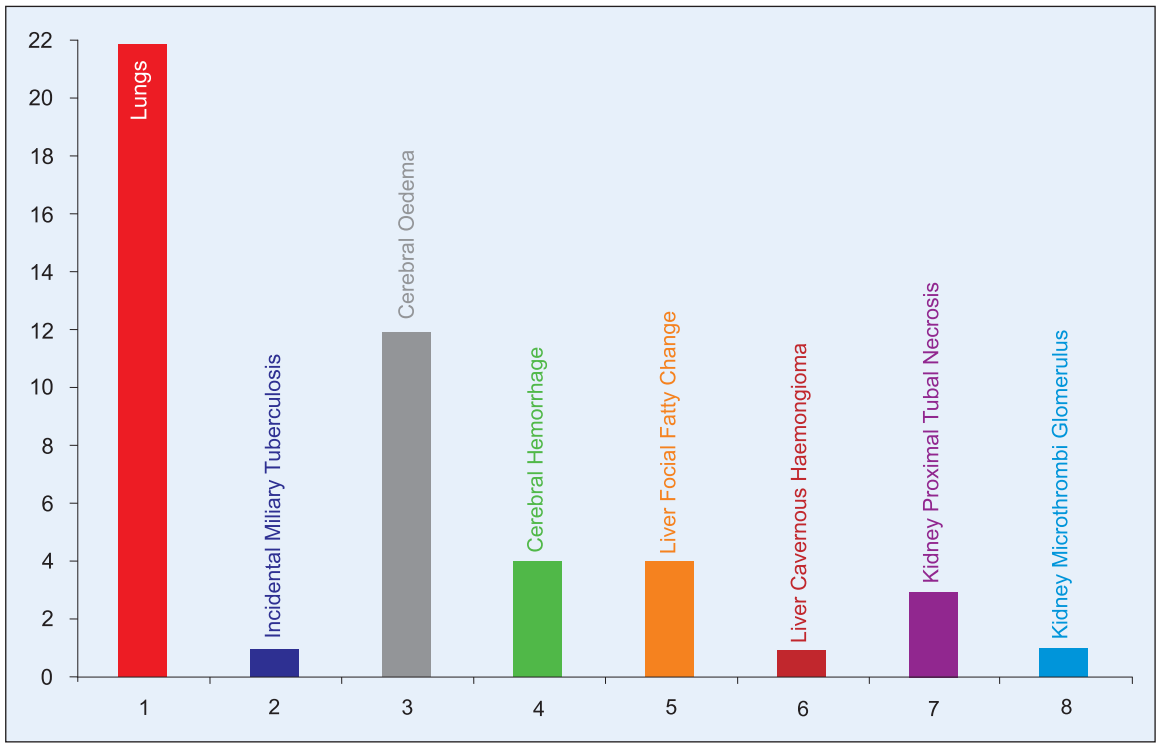

Figure 4.2. Series II: Overall Prevalence of Extra-Pulmonary Lesions

Summary of Histopathological Findings

Although many organs were affected, the most prominent findings were in the lungs. In the early series, there was a gross increase in the weight of the lungs, nearly 3 times that of the normal. The entire respiratory tract showed a series of pathological changes. There was intense congestion and denudation of the epithelium of the trachea and the major divisions of the bronchi. There were foci of ulceration. The lungs were heavily water-logged and had the characteristic cherry red colour. Microscopically there were extensive changes such as necrotizing bronchiolitis and widespread

damage of the lung parenchyma. The major findings were those of acute bronchiolitis, bronchopneumonia, pulmonary hemorrhages, and pulmonary edema, with outpouring of albuminous fluid into the alveoli, pneumonitis and alveolitis. The histo-pathological changes in other organs were suggestive of extensive or widespread cerebral, edema or swelling, peri-capillary ring hemorrhages, both in the cortex as well as the white matter. The liver in a number of cases showed varying degrees of fatty change. The kidneys showed marked congestion in most of the cases and tubular necrosis in some. The gastro-intestinal tract showed mesenteric and sub mucous hemorrhages, and necrotizing enteritis. The heart muscle showed generalized edema without obvious necrosis.

A subsequent group of autopsies on victims who died during the 8-12 weeks of the episode revealed less marked but essentially similar picture of pulmonary changes. But there was no suggestion of any interstitial or parenchymal fibrosis. The observations of cerebral edema and anoxia were consistently prominent even in the later stage.

Electron Microscopic Studies

Ultra-structural studies of representative samples of tissues at different periods showed a consistent reduction, a loss of Type I pneumonocyte representing the squamous epithelial cell which lines the alveolar surface. This could account for impairment of trans-alveolar exchange of blood gases due to the loss of pulmonary surfactant. By contrast, the Type II pneumonocyte, the precursor of pulmonary surfactants, with characteristic multilayered lamellar concentric bodies, was found consistently within the alveolar spaces. Electron microscopy of the brain sections also confirmed the histological finding of cerebral edema and neuronal degeneration. Neurons showed depletion of Nissl granules, dilatation of the Endoplasmic Reticulam and marked swelling of peri-capillary astrocytes. Flocculent opacities in the mitochondria, presaging cellular death, were seen quite frequently in sections from the heart muscle, liver cells and nerve cells of the brain.

In brief, the autopsy studies at the Gross, Light and Electron Microscopic level, revealed a wide spectrum of immediate and long-term histo-pathological changes with possible functional impairment amongst the survivors. The details of the Histopathological & Toxicological findings were presented at several Conferences, notably The 1st International Symposium on IsoCyanates at Stockholm (***Annexure 4.1***) in 2000 and The 4th Sir Dorabji Tata Symposium on: Acute Respiratory Disease at Bangalore in 2003 and subsequently published on Editor's request in Current Science of 2004 (***Annexure 4.2***).

Lung Biopsy Studies

The issue of performing lung biopsies in the prevalent situation of Bhopal was discussed in great detail in a couple of meetings organized in Bhopal and also in Delhi under the auspices of Bhopal Gas Commission. Although, there were a few preliminary studies, it was decided to defer the program in the surcharged situation. However, a brief mention is being made to recall the findings:

Six to Eight months after exposure, Dr. Darbari and associates in 1985, undertook 'open lung biopsies' in 4 patients which showed the characteristic feature / picture of ***bronchiolitis obliterans*** of a fairly large sized terminal bronchiole. The surrounding lung parenchyma showed marked inflammatory exudates. Histology of all the cases showed "thickening of alveolar septae with diffuse interstitial fibrosis with collagen. Neutrophils, eosinophils and plasma cells were consistently absent and macrophages could be seen in the alveolar lumen. The terminal bronchioles were inflamed with presence of exudates in their lumen. Mild to moderate pulmonary hypertension was suggestive from the thickened arterial walls. Two of the four cases presented were those of interstitial fibrosis diagnosed on clinical, radiological and Pulmonary Function Tests.

Later on, Kamat et al (1987) performed open lung biopsies in three patients and observed similar changes: (a) Sub-Pleural & Septal Fibrosis, (b) Interstitial Cellular aggregates, (c) Compensatory Emphysema, (d) Bronchiolitis and (e) Peri-bronchial & Peri-vascular Fibrosis.

Long-Term BAL (Broncho Alveolar Lavage) Studies

Apart from large number of deaths in Bhopal due to acute pulmonary edema, persistent respiratory symptoms in the survivors raised suspicions of development of interstitial lung disease in some of the survivors. As already stated,

performing lung biopsies was not pursued in the Bhopal scenario. Similarly, Lung Function Tests also seemed to have certain limitations. As an alternative, Dr. Vijayan and associates thought that BAL studies might be of help in the resolution of issues related to post-exposure 'alveolitis'. In the process, it has been possible to distinguish the two categories of long-term 'macrophage reaction'. Patients accustomed to smoking revealed superimposition of 'neutrophil reaction'. The initial one-point study was re-confirmed in the by follow-up BAL study. The detail of the first study is presented as ***Annexure 4.3***, the follow-up study published year and half later was published in Respiratory Medicine (1995) by Dr. Vijayan.

In 1987, Kanhere et al. also studied the Morphology of Placenta in 134 cases of 'pregnant women exposed to Bhopal Gas leak and concluded that while no significant differences were observed in full-term deliveries as between Gas Exposed & Control groups. 'Hydropic degeneration' of the placenta was seen in the cases that had undergone medical termination of pregnancy.

Experimental Studies with MIC & Related Chemicals

Until the occurrence of the Bhopal Gas Disaster, practically nothing was known about the toxicity of MIC. Even subsequently, many of the initial experimental studies were related to simulation of the irritant effects of repeated exposures, rather than investigating the several parameters inherent to Bhopal Disaster such as: age of the affected victim, severity of exposure due to dosage variation, long-term follow-up after single exposures to the main chemical MIC, and its aqueous derivatives like Methyl Amine (MA) & Dimethyl Urea (DMU). Jeevaratnam and Sriramachari undertook a comprehensive Experimental study at DRDE, Gwalior involving variable dosages and periods of study times, which answered several related issues. The studies were published initially in the IJMR (***Annexure 4.4***), followed by Archives of Toxicicology in 1994a & 1994b, discussed in detail in ***Annexure 4.2*** and also presented at the 1st International Symposium held in Stockholm in 2000 (***Annexure 4.5***).

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Pathology and Toxicology of Methyl Isocyanate and MIC Derivatives in Bhopal Disaster

Samavedam Sriramachari[1] & Heeresh Chandra[2]

In any consideration of the "Biological Effects of isocyanates", the worst-ever chemical disaster in Bhopal consti tutes a. unique landmark and role model in several respects.

On the midnight of 2nd December 1984, following a runaway chemical reaction, nearly 40 tons of methyl isocyanate (MIC) was released into the atmosphere over a densely populated area of Bhopal City in India. Over 3,000 people died and many more were condemned to long-term morbidity. Apart from MIC, the aerosol of toxic gases inhaled by the victims possibly contained a. mixture of aqueous and thermal decomposition products, and a host of reactant chemicals and polymers of MIC. Detailed autopsy studies and chemical investigations were carried out, the salient features of which are given below:

Autopsy Studies - Gross Anatomy & Histopathology

Initial autopsy studies during the first four weeks revealed a characteristic "cherry red discoloration" of lung, the primary target organ, alongside massive pulmonary edema, emphysema and hemorrhages, generalized visceral congestion, cerebral edema, ring hemorrhages and anoxic brain damage.

Extensive pulmonary & exudative changes were observed during subsequent autopsy studies carried out on victims succumbing one to four months post-exposure. Later studies from four months to one year and beyond revealed diffuse interstitial pulmonary fibrosis (DIPF)

Issue of Cyanide Toxicity

In over 100 autopsies of instantaneous and early deaths, we observed "arterialization of blood and a 'cherry red' discoloration of the lungs". This singular feature aroused a genuine suspicion of possible "cyanide toxicity", due to release of a certain quantity of HCN during "pyrolytic decomposition of MIC" (Blake and Ijadi-Maghsoodi, 1983) Although Union Carbide stoutly deprecated the possibility of HCN release, our subsequent chemical analysis confirmed an elevation of blood cyanide level.

Initial investigations like "Blood Gas Studies", elevated 2-3 DPG levels and a negative spectroscopic evidence for carboxy~hemoglobin lent further support to an unknown type of "histo-toxic anoxia" due to MIC. While Union Carbide advocated that MIC does not cross the "alveolar-capillary barrier", our findings of elevated levels of blood cyanide and demonstration of MIC in the blood of victims are significant.

Evidence in support of Cyanide Toxicity

Direct evidence: In a study comprising 128 samples, a considerable elevation in the blood cyanide levels was observed in the living as well as dead persons exposed to MIC, as compared with the unexposed.

Indirect evidence: The dramatic "Clinical Response" to administration of I.V. Sodium Thiosulphate (NaTs) was followed by a few successful "Double Blind Clinical Trials",

Several clinical & epidemiological studies were carried and it was found that in many of the 'exposes' the urinary thiocyanate level was raised above the normal level of 1 mg%. Immediately following NaTs, there was a further increase, indicating the accumulation of the cyanide metabolites. Over 18,000 samples were monitored for the procedure over a period of '18 months of follow-up, when the metabolic disturbance tapered off.

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Binding of MIC molecule to the free amino group of the end-terminal amino acids of hemoglobin and other tissue proteins

Further, in the annals of chemical disasters, the Bhopal episode appears to be the first instance, where the offending chemical/s have been tracked down to the bodies of the victims. The presence of MIC itself was established by the demonstration of "N-Carbamoylated end-terminal valine residues of hemoglobin (Hb)" in the blood samples from cadavers and 'survivors', Binding of MIC to the terminal valine residues of Hb or Myoglobin (carbamoylation) could explain the cherry red color transformation and functional disturbances, like impaired transport of carbon dioxide, release of oxygen and extreme muscle weakness.

Initially, a reduction of "free amino groups" of hemoglobin was demonstrated by the TNBS reaction Subsequent chromatographic studies for demonstration of Methyl Valine Hydantoin (MVH) confirmed that MIC was bound to end-tem1inal valine residues. By mid-January 1985, we detected v MVH in 19 out of 60 exposes and 7 out of 11 cord blood samples. Thus, it has been shown that MIC not only crosses the alveolar-capillary but even the placental barriers. Depending on the extent of binding and the continuous formation o(new blood, the risk seems to be "contained".

"Methyl hydrations" of ten other end-terminal amino acids were identified using G.C.-M.S. equipped with ion trap detector, in the several viscera, of victims who survived up to 16 months. Unfortunately, as mentioned earlier, the mechanism of random distribution of MIC throughout the body eluded us, because of lack of facilities to tackle S-glutathione and several enzyme systems containing sulphydryl of groups. Later Bailie & Slatter (1991), demonstrated S-Carbamoylation in experimental material using a chemical ionization detector. However, the possibility of reversible S-Carbamoylation of SH groups of rhodanese, aldolase and cholinesterase, still remains unanswered!

Comparative Forensic Toxicological Investigation of the "tank Residue" and tissue extracts of cryo-preserved viscera from autopsied cases

A comparative G.C-M.S. study of the "incriminated tank-residue" and cryo-preserved autopsy tissue-extracts revealed an overlapping spectrum of MIC derivatives. Undoubtedly, the information is of considerable "evidentiary value". But more importantly, the high molecular weights, of some of the new compounds, may have a bearing on the controversial issue as to the maximum temperatures attained during the "run-away chemical reaction" which could have favored the release of HCN. Moreover, the possible toxicity of some of the unknown compounds with high molecular weight (MW) still remains an enigma.

Using G.C.-M.S (ITD) technique we detected a total of 21 MIC-derived chemicals in the milieu-exterior as well as -interior systems. Four of these compounds had a MW greater than 150 indicating higher Melting Points and a high range of temperatures which operated during the "run-a way-chemical reaction". Such information if confirmed might explain whether the reaction could have promoted pyrolysis of MIC and release of HCN.

Thus the scientific studies have provided acceptable and convincing answers to several issues raised by the Bhopal disaster involving methyl isocyanate.

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The Bhopal Gas Tragedy: An Environmental Disaster

S. Sriramachari

The multi-disciplinary study of histopathology and toxicology of Bhopal gas tragedy resolved several issues. First, the progression of severe pulmonary oedema to chronic fibrosis was confirmed experimentally, following a single exposure to MIC. Analysis of the residue in Tank 610 revealed over 21 chemicals. Apart from MIC and HCN, some of them were tracked down to the blood and viscera of dead and living 'exposees'. The rationale of NaTS therapy was substantiated by elevated urinary NaSCN levels in Double Blind Clinical Trials as well as patients. Apart from cyanide, the 'cherry red' discolouration was also shown to result from binding of MIC to end-terminal valine residues of Hb, as shown by changes in 2–3DPG levels and blood gas profiles. The finding of Ncarbamoylation of several other end-terminal amino acids of tissue proteins confirmed the distribution of MIC within the body, although the underlying mechanism is not yet fully understood. Possibly, the much faster S-carbamoylated compounds of the blood like glutathione and other sulphydrylcontaining enzymes like rhodanese could be responsible for re-circulation of MIC and protracted cyanide toxicity. It is hoped that eventually the enigma of the 'Bio-chemical Lesion' of MIC toxicity will be unraveled.

BY all accounts the Bhopal gas leak on the night of 2–3 December 1984, is the worst chemical disaster in history. It took a heavy toll of human lives. People started dying within hours and more than 2000 lives were lost in the first few days. Late Heeresh Chandra, the man who had to deal with it first-hand, presented the forensic aspects at the Third Indo-Pacific Congress on Legal Medicine held at Madras in December 1989, followed by Sriramachari on the histopathology and toxicological studies. According to Ivor Doney who reviewed the proceedings, 'A silent stunned audience listened with awe the terrible story of 1984, when on one tragic day poisonous fumes killed hundreds of people or maimed thousands of them in the ensuing months'. He likened this tragedy to 'Pompeii suddenly engulfed in the dust of Vesuvius, or Hiroshima when the atom bomb was dropped'. He concludes 'that the story should be told at some future international forensic meetings again and again and again'.

By 7.00 AM, 70 people were dead, by 9.00 AM 260 were dead and thereafter the figures continued to rise. Though not all dead bodies were brought to the Medico-Legal Institute (MLI), 311 bodies were received on 3.12.1984, followed by another 250 on 4.12.1984. Thereafter, the rate declined. A total of 731 bodies were received in December 1984 alone, 103 in 1985; 90 in 1986 and 44 and 22 respectively in 1987 and 1988. These figures from the morgue may not account for all the deaths in the city of Bhopal. The MLI continued to perform autopsies on gas-affected victims in subsequent years.

Brief clinical manifestations

According to Dureja and Saxena[1], one of the earliest of the 'Rescue Teams' to reach Bhopal when panic was at its peak at major hospitals of the city, the patients could be graded symptomatically into four categories: (i) Minor eye ailments, throat irritation and cough, (ii) Severe conjunctivitis, keratitis, acute bronchitis and drowsiness, (iii) Severe pulmonary oedema leading to cardio-respiratory distress, and (iv) Convulsions, followed by cardio-respiratory arrest. Intense fatigue and muscular weakness was another common feature. Another early and comprehensive report is by Kamat *et al*.[2]. Soon thereafter, other clinicians like N. P. Misra[3], P. S. Narayanan, and S. K. Jain had encountered similar patterns, with minor variations. Several thousands survived with a variety of morbidity and permanent disabilities. According to the recent press reports, there are a large numbers of survivors, with lingering ailments and incapacity to work.

Non-availability of any information about the toxicity of even the parent compound, MIC (methyl isocyanate), was a great impediment to institute 'detoxication measures' and lay down guidelines for therapeutic intervention and management of the victims. Hence, there was an urgent need to generate *de novo*, authentic scientific evidence and information. The ICMR rushed to the scene and tried to fill the void. It funded, amongst 24 other projects, two comprehensive studies on toxicology and a collateral

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project on PFT (pulmonary function tests)/blood gas analyses. Both these projects rapidly transformed themselves into 'multidisciplinary and multi-institutional investigations' (Tables 1 and 2).

Nature of the toxic gases in Bhopal

Public attention to the high reactivity of MIC was drawn by Devkumar and Mukherjee[4]. It was followed by more authoritative accounts in the Varadarajan Committee Report5. According to the report, there was a massive leak of MIC stored for a long period in the incriminated Tank 610 of the Pesticide Plant of Union Carbide of India Ltd (UCIL). Several hypotheses attributed for this disaster include, 'prolonged bulk storage of 42 tons of MIC, nonfunctioning refrigeration system, failure of safety measures and malfunctioning of neutralization facilities'. One or more of these factors might have contributed to the accidental and uncontrolled runaway reaction. From all accounts, quite unlike leakage of a single chemical like ammonia, sulphuric acid, phosgene or hydro cyanic acid, the gas release in Bhopal is not due to mere leakage of cold MIC. The presence of an array of multiple chemicals was demonstrated[5–7]. Careful re-examination by the toxicology project of the ICMR, revealed the presence of as many as 21 chemical constituents, including 9 or 10 additional unidentified compounds[8]. However, the toxicity was NOT known about any of the compounds, including MIC.

Table 1. Multidisciplinary studies undertaken

Human autopsies
Histopathology
Electron microscopy
Experimental studies
Pulmonary function tests (PFT)
Elevated levels of haemoglobin (Hb)
Alterations in blood gases
Elevated 2–3 DPG (di-phospho-glycerate) levels
Evidence of acute and chronic cyanide toxicity
Therapeutic response to detoxification by NaTS
Forensic toxicology
Proof of direct binding of MIC to Hb and tissue proteins through N-carbamoylation
Faster and reversible S-carbamoylation (attempted but not successful)

While elevation of pressure and temperature inside the closed tank or container in Bhopal was widely recognized, the liberation of hydrogen cyanide (HCN) became one of the most contentious issues. In retrospect, it is amusing that the Union Carbide Corporation (UCC) decried cyanogensis and let loose a campaign of misinformation. There were sufficient indications to that effect even in UCC's earlier reports[9]. And it was amply confirmed in the publication of Blake and Ijadi-Maghsoodi[10], about two years prior to the Bhopal disaster. While there is no doubt about the decomposition of MIC, it was suppressed on puerile grounds, due to uncertainty of the temperature attained within Tank 610 and in the absence of consensus as to the quantity of HCN liberated at specific temperatures, say 200°C, 300°C and 420°C! Further, it was known, ever since 1927, that apart from other nitrile compounds, HCN forms an 'adduct with MIC' itself[11]. Depending on the reactions, theoretically it is even possible for the more lethal 'cyanogen' to be formed. It is proposed to discuss later several issues related to confirmation of 'cyanide toxicity in Bhopal'.

Critical human autopsy studies

Heeresh Chandra and his colleagues started performing autopsies within 72 h of the disaster. It was noticed that the usual post-mortem lividity or cyanosis was not present; instead, there was a pinkish discolouration over all parts of the body.

Table 2. Trans-institutional collaboration

Institutions	Collaborators
Medico-Legal Institute, M.G.M. College, Bhopal	Heeresh Chandra, Satapathi, colleagues and scientific project staff
Department of Pathology, M.G.M. College, Bhopal	B. S. Darbari and S. Kanhere
Institute of Pathology-ICMR, S.J. Hospital, New Delhi	S. Sriramachari and H. M. K. Saxena, Ashok Mukherjee and A K Jain
G.B. Pant Hospital, New Delhi	P. S. Narayanan
AIIMS, New Delhi	A. Ramaiah and Roman Reddy
DRDE, Gwalior	P. K. Ramachandran and colleagues, K. Jeevaratnam
INMAS, New Delhi	N. Lakshmipathi, S. K. Sharma and Pant
DIPAS, New Delhi	K. Sridharan and A. C. Patil
IIT, Madras	D. V. Ramana
IICT, Hyderabad	M. Vairamani

The most important findings were in the lungs. There was a gross increase in the weight of nearly 2 ½– 3 times the normal. The entire respiratory tract showed a series of pathological changes. The lungs were heavily water logged and had a distinctive cherry-red colour (Figure 1 *a*). The mucosa was intensely congested. The trachea and the major divisions of the bronchi revealed necrotizing or ulcerative changes. The following striking microscopic findings were noticed. Severe tracheitis and bronchitis with denudation of the epithelium was seen in some sections (Figure 1 *b*). There was marked congestion and thickening of the alveolar septa (Figure 1 *c*). The alveoli were filled with eosinophilic albuminous fluid (Figure 2 *a*).

Intra-alveolar hyaline membranes were seen frequently. Many sections of the lung showed extensive emphysematous areas (Figure 2 *b*). There was very little evidence of secondary infection. In some places there was necrotizing bronchiolotis (Figure 2 *c*). Polymorpho-nuclear cellular infiltrates were not very prominent. Instead, cellular response in the lung was largely one of proliferation of the alveolar macrophages.

More or less similar features were observed in a large number of autopsies carried out in the second week following the gas leak. Both grossly and microscopically, the lungs continued to be the seat of primary change. The lung parenchyma showed varying degrees of bronchiolitis,

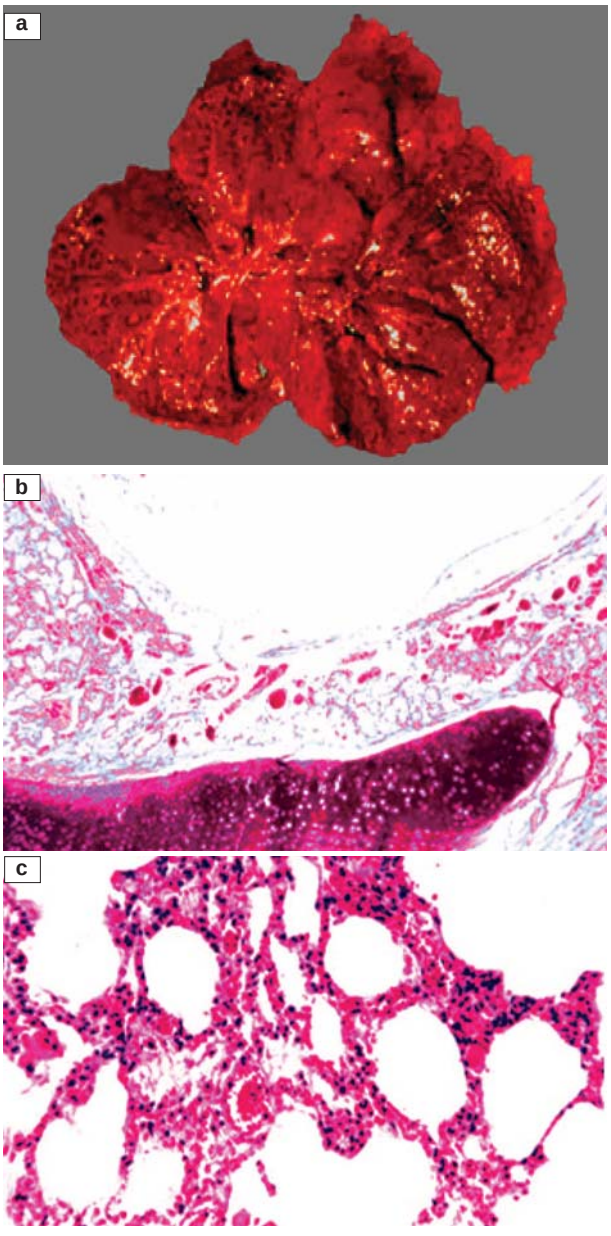

Figure 1 *a*–*c*. Early autopsy findings. ***a***, Gross picture of lung shows typical cherry red discolouration; ***b***, Low power photomicrograph showing marked congestion and epithelial denudation of trachea; ***c***, hotomicrograph of lung parenchyma showing marked congestion and thickening of alveolar septa.

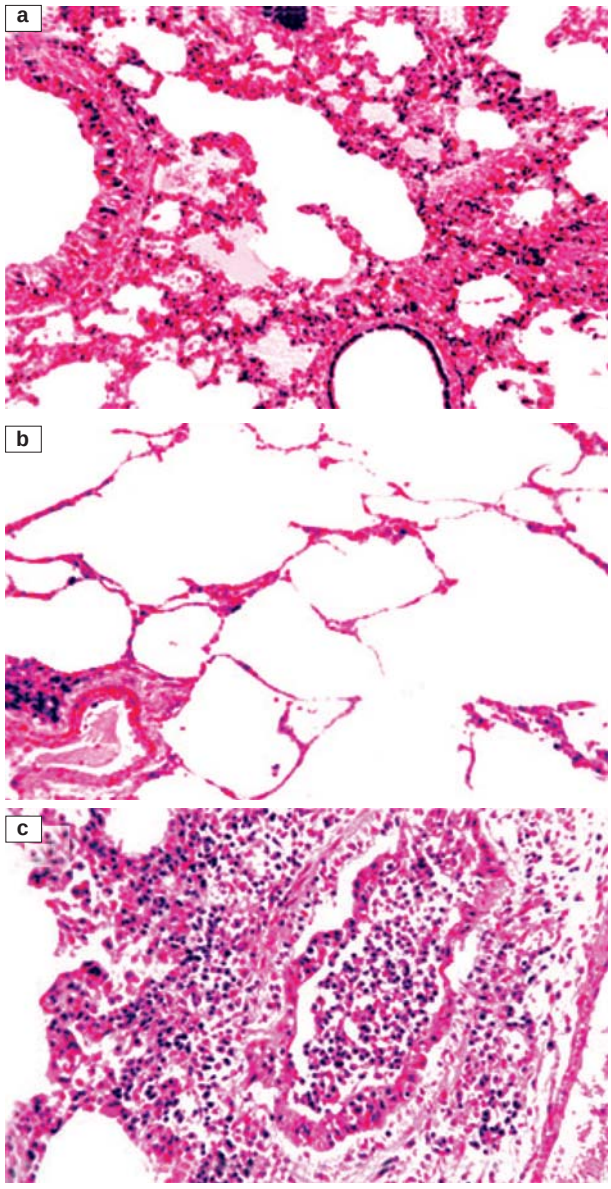

Figure 2 *a*–*c*. Early autopsy findings. ***a***, Photomicrograph showing terminal and respiratory tubules, interstitial congestion and intra-alveolar oedema and some emphysema; ***b***, Photomicrograph of sections of lung with bullous emphysema howing destruction of alveolar septa and coalescence; ***c***, Photomicrograph shows peribronchial and intraluminal bronchiolitis suggestive of necrotizing bronchiolitis.

bronchopneumonic changes and infiltration of the alveolar spaces by polymorpho-nuclear cells, around and within the lumen of bronchioles (Figure 3 *a*). There was a gradual transition in the pathological changes. There was persistence of acute desquamative changes in the trachea and the main divisions of the bronchi. The alveolar spaces also showed a variable degree of infiltration by inflammatory cells.

In the acute phase, the other viscera showed gross appearance of oedema of the brain and congestion of the leptomeninges. In a few cases, the liver showed mild degree of fatty change, which might be either incidental or secondary to severe shifts in the intra and extra-cellular fluid, apparently associated with outpouring of fluid into the pulmonary parenchyma. A noteworthy feature in the earlier series of autopsies was the marked distension of the gall bladder with excess of fluid. Curiously enough, analogues increased excretion of bile and water content of excreta has been reported[12] in chicks fed 'cassava rich in cyanide' content.

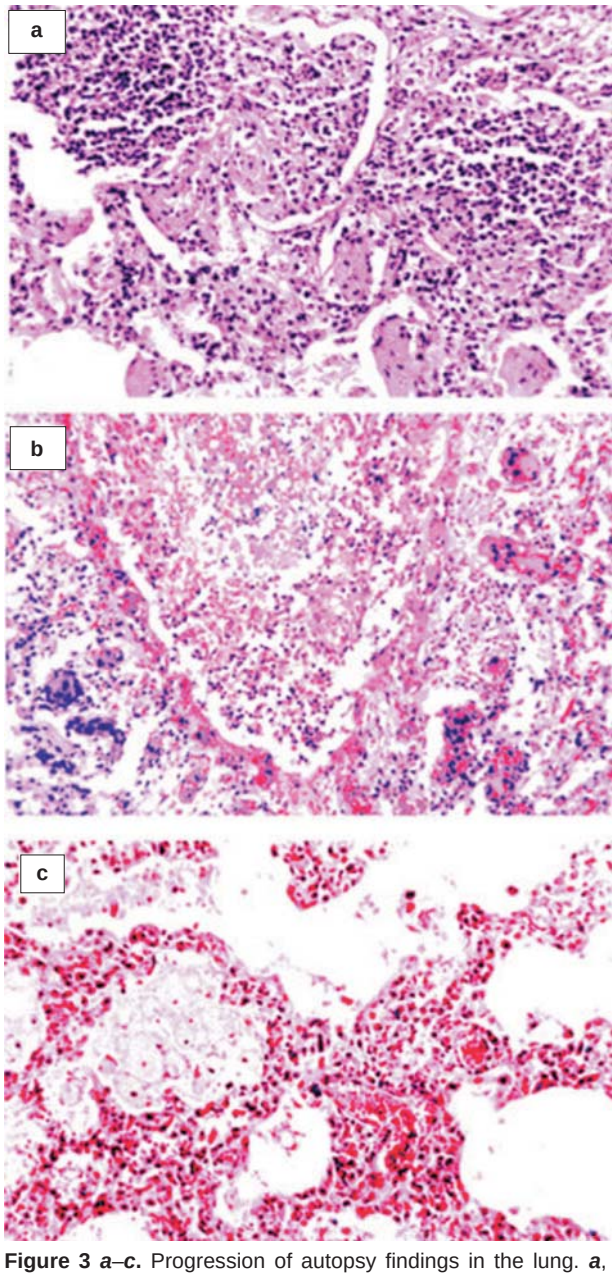

Figure 3 *a*–*c*. Progression of autopsy findings in the lung. ***a***, Photomicrographshowing picture of early bronchopneumonic consolidation; ***b***, Photomicrograph showing atypical picture of *bronchiolitis obliterans*; ***c***, A later autopsy shows a picture of congestion and interstitial pneumonitis with intra-alveolar macrophages.

Continuing autopsy studies

Excluding the initial lot of 22 cases studied in 1984, the Institute of Pathology received during 1985–88, representative tissue samples from over 170 autopsies. Approximately there were 90 cases in 1985; 18 in 1986; 17 in 1987 and 47 in 1988, although there is a possibility of some overlap in the dates.

In 1985 the earlier picture of pulmonary oedema, bronchopneumonia and bronchiolitis seems to be gradually replaced by a picture of diffuse interstitial pneumonitis, but without any significant fibrosis. There was progressive decrease of oedematous fluid accompanied by well-defined interstitial mononuclear cellular reaction, histiocytosis and macrophages. At this stage, no significant changes indicative of passive venous congestion were present.

The autopsy material obtained in 1986–87 showed a picture of interstitial pneumonitis with thickening oedema and increased cellularity of the alveolar septae progressing to more organized fibrosis. Apart from the presence of 'bronchiolotis obliterans' in an occasional field (Figure 3 *b*), there was evidence of 'desquamatative interstitial pneumonitis' (DIP) (Figure 3 *c*). An occasional case showed evidence of 'giant cell interstitial pneumonitis' (GIP) (Figure 4 *a*) of the usual or giant cell type. A unique autopsy case of a young doctor exposed to toxic gases on 3.12.1984 who died in the middle of 1987, revealed significant findings. Multiple sections of the lung showed broncho-pneumonic changes, superimposed with a picture of desquamatative alveolitis with large collections of macrophages. (Figure 4 *b*). Normal or functional alveoli were scarcely seen in most of the sections. There was evidence of atelectasis with intervening pulmonary fibrosis. The findings were suggestive of marked interstitial fibrosis or 'fibrosing alveolitis' (Figure 4 *c*). Yet another significant observation in 1986–87 is the picture of chronic passive venous congestion of the liver with characteristic features of congestion, necrosis and pigmentary changes indicative of right heart failure.

The material received in 1988 showed a similar picture, but of a more organized nature. There was greater fibrosis of alveolar septae and patchy fibrosis of lung parenchyma.

Histopathological changes in other organs

The brain, in general, showed in the acute phase hallmarks of gross oedema and congestion of the leptomeninges. In a few cases there was obvious evidence of sub-arachnoid and intra-ventricular haemorrhages (Figure 5 *a*). Occasional cases also showed grossly cortical and sub-cortical ring haemorrhages (Figure 5 *b*). Miscrospically distinct pericapillary and 'focal flame haemorrhages' were seen in the cortex as well as the white matter (Figure 5 *c*). The brains in general showed evidence of generalized pericellular and peri-capillary oedema. It was more apparent in the paraffin sections (Figure 6 *a*). The same was also confirmed in the celloidin sections. Focal acute nerve cell degeneration of Nissl was seen in a scattered manner in the dentate nucleus (Figure 6 *b*). However, there was no indication of satellitosis or neuronophagia. Selective neuronal damage of Purkinje cells was seen in the cerebellar folia (Figure 6 *c*).

The liver showed a picture with normal appearance in most of the cases, with some degree of moderate fatty change in a few cases and necrosis and disorganization of the liver cell cords occasionally.

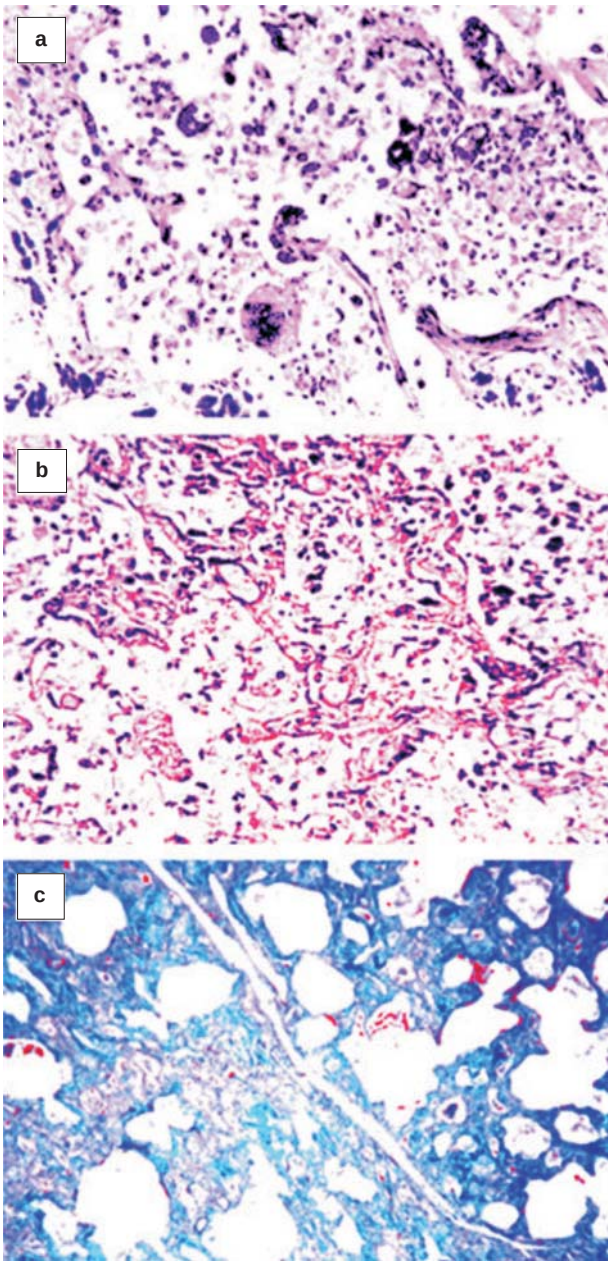

Figure 4 *a*–*c*. Late autopsy findings. ***a***, Photomicrograph showing an occasional picture of giant cell interstitial pneumonitis; ***b***, Photomicrograph showing a picture of bronchopneumonia superimposed by desquamative alveolitis; ***c***, Photomicrograph of the same case showing extensive atalectasis and fibrosing alveolitis (Masson Trichrome Stain).

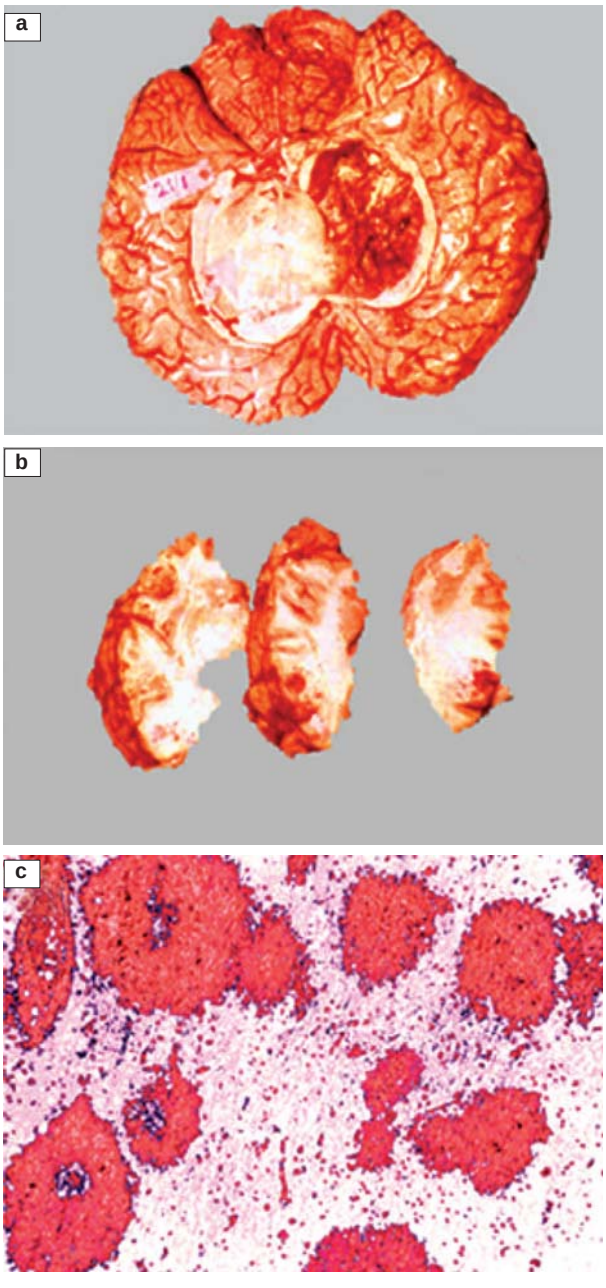

Figure 5 *a*–*c*. Autopsy findings in other organs. ***a***, Gross appearance in early autopsy with marked sub-arachnoid congestion and intraventricular haemorrhages; ***b***, Cortical and sub-cortical haemorrhages in gross sections of the brain seen occasionally; ***c***, Photomicrograph of the brain showing peri-capillary flame haemorrhages.

The kidney showed in a few cases acute tubular necrosis, particularly of the proximal tubules. The medulla in such case showed congestion and presence of tubular casts in the collecting ducts.

Ultrastructural Changes

The study indicated certain interesting changes, which supported the light microscopic findings. The alveoli of the lungs showed a consistent picture of thickening of the alveolar membrane. Although there was a marked reduction of the epithelial lining or Type-I pneumonocytes (Figure 7 *a*), it is significant that Type-II pneumonocytes were present in large numbers as electron-dense whorl-like material (Figure 7 *b*). This probably represents an attempt at enhanced synthesis of the surfactant material phosphatidyl ethanolamine. There was no evidence of acute myocardial infarctionor myolysis except for the presence of oedema. Electron microscopic examination of heart muscle tissue revealed the presence of degenerated mitochondria with floccular

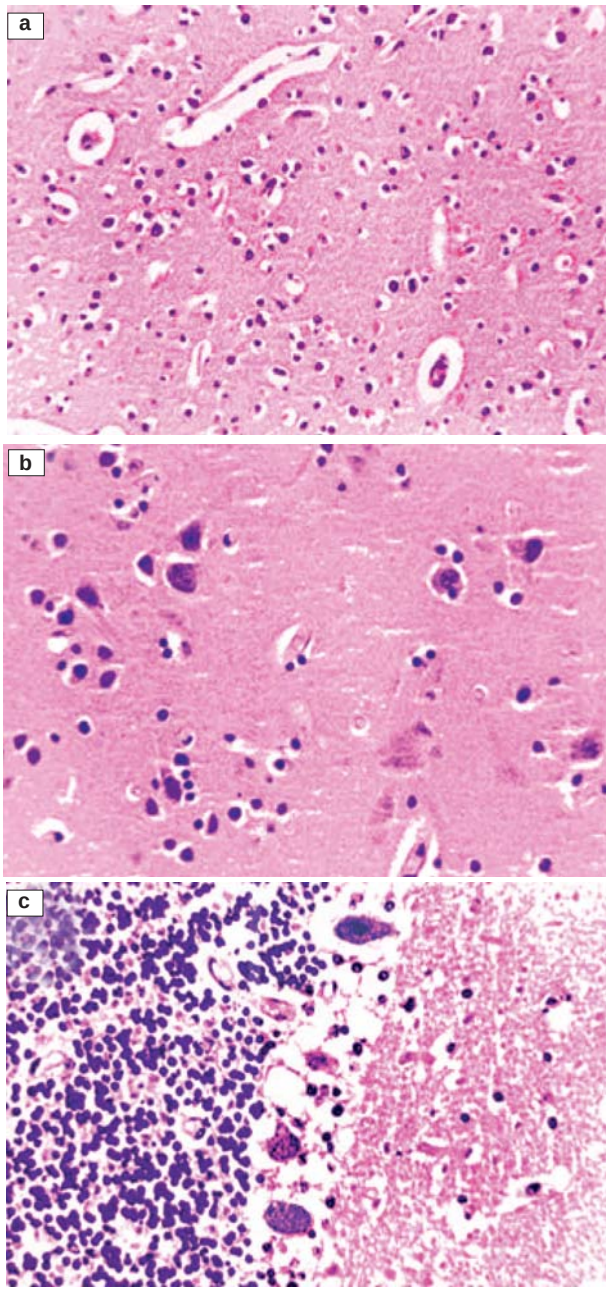

Figure 6 *a*–*c*. Autopsy findings in the brain. ***a***, Photomicrograph showing peri-cellular and peri-capillary oedema suggestive of cerebral anoxia; ***b***, Photomicrograph showing focal acute nerve cell degeneration of dentate nucleus of cerebellum suggestive of selective neuronal damage due to anoxia; ***c***, Photomicrograph of typical anoxic changes with the partial loss of Purkinje cells of cerebellar folia.

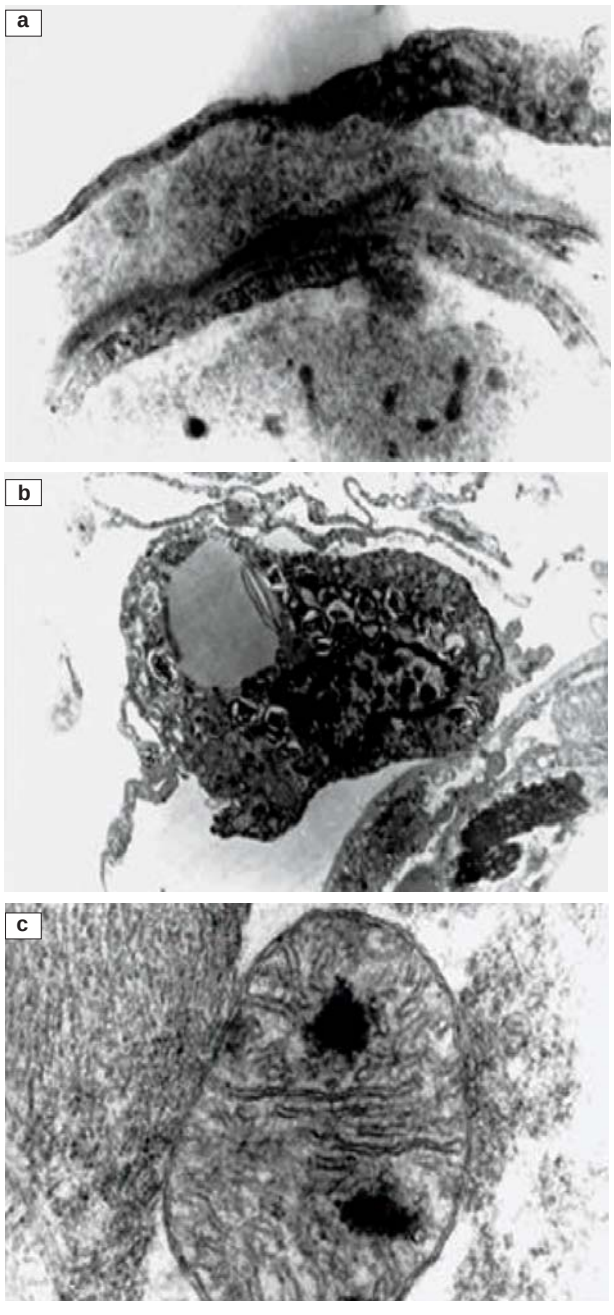

Figure 7 *a*–*c*. Early ultra structural changes. ***a***, Alveolar septa showing reduction of type I pneumonocytes; ***b***, Electron icrograph showing increase of type II pneumonocytes with whorl-like lectron dense material suggestive of increased production of surfactant; ***c***, Electron micrograph of mitochondria of cardiac tissue showing floccular degeneration.

masses (Figure 7 *c*). However, it is to be pointed out that these ultra-structural findings were seen sparsely.

Electron microscopy of the brain tissue also confirmed the enlargement of pericapillary Virchow–Robin spaces (Figure 8 *a*) and swollen oligodendroglial cells and astrocytic processes abutting on the pericapillary gliovascular membranes (Figure 8 *b*). Interestingly, some of the erythrocytes within the capillaries were electron lucent (Figure 8 *c*). This is suggestive of leaching out of some lipid substances of the erythrocyte membrane, possibly due to loss of carbamoylated glutathione.

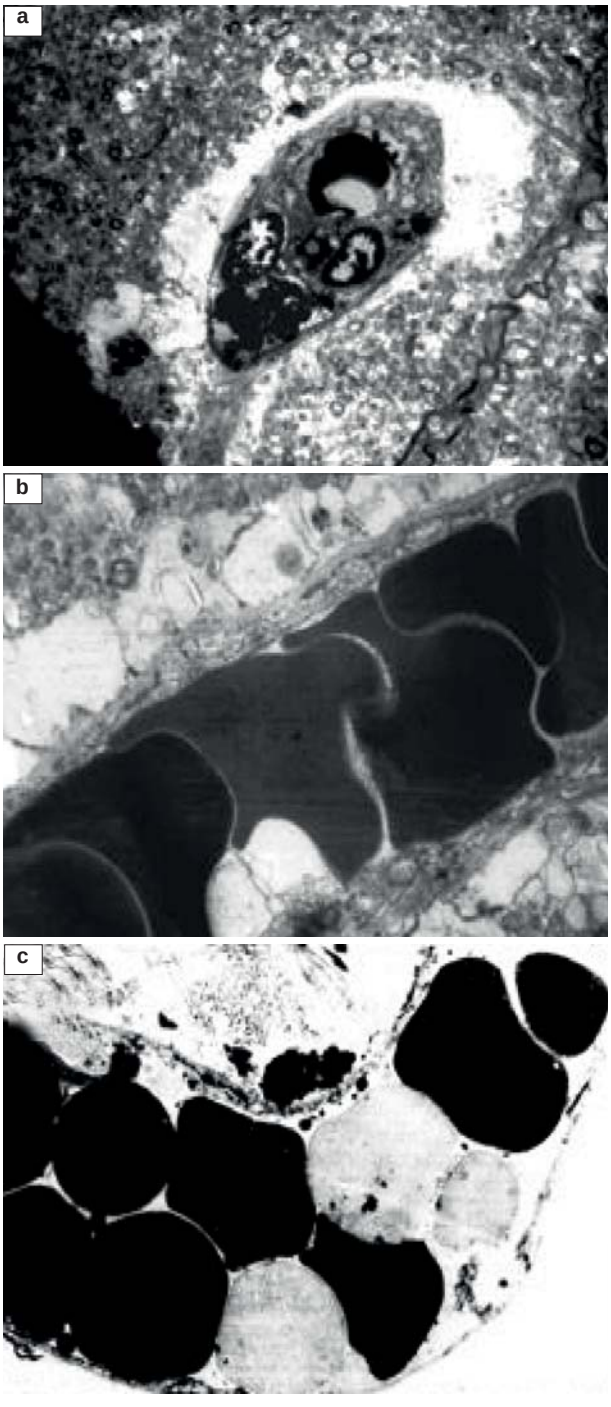

Figure 8 *a*–*c*. Early ultra structural changes. ***a***, Electron micrograph showing enlargement of Virchow–Robin pericapillary spaces indicative of extensive oedema; ***b***, Electron micrograph of another area showing capillary packed with erythrocytes and oedematous changes in the gliovascular membrane in astrocytic processes and oligodendroglia; ***c***, Electron micrograph of a capillary structure in the brain showing electron lucent erythrocytes suggestive of loss of some material.

Experimental studies

As pointed out earlier, practically nothing was known about the toxicity of MIC, except for some acute experiments conducted by UCC in the Bushy Run Laboratories. In the aftermath of the Bhopal gas leak, several experimental studies were undertaken in different parts of the world. But most of them dealt with the study of its irritant effects on repeated exposures. Only Nemery *et al.*[13] attempted to study the effects of single exposure, although confined only to the acute phase. Further, it was considered desirable to know about the toxic effects of not only pure MIC, but also its aqueous derivatives, methyl amine and di-methyl urea with a view to simulating the *in vivo* situation. Hence, the pathological effects of a single exposure of rats to the above three constituents were also studied. Apart from the lethal damage, the sub-lethal effects were also studied. In addition to the inhalation route with single or two LC_{50} doses, the toxic chemicals were administered subcutaneously at one LD_{50} dosage. Only surviving animals, sacrificed at various time intervals, extending from one day to ten weeks, were submitted to histopathology. Care was taken to discard the dead animals, with attendant autolytic changes. In all, lungs from over 240 animals were available for study. With the help of this experiment, it was possible to reproduce the full spectrum of histopathology of lung, seen in the human victims. Some of the B&W illustrations published[14,15] have since been reproduced as colour photomicrographs. Initially, at the end of 24 h there was an overwhelming bland oedematous fluid filling up of the alveoli and eosinophilic necrosis of the bronchial epithelium (Figure 9 *a* at one LC_{50} and Figure 9 *b* at two LC_{50} dosages). Even when administered subcutaneously at a dosage of one LD50, the lung was found to be the target organ with intense interstitial pneumonitis although without any noticeable oedema (Figure 9 *c*). This is in consonance with the so-called 'special vulnerability' with alveolar membrane by the inhalational route as shown earlier in the case of 'phosgene' (cited by Cohen and Oppenheimer[16]). At subsequent periods, the sequaelae of intra-alveolar oedema and interstitial pneumonitis, and bronchitis and even fibrosis were observed by the end of one week (Figure 10 *a* and *b*) and with both the routes of administration. Even diffuse pulmonary fibrosis could be demonstrated by the end of

ten weeks (Figure 10 *c*). Thereby, it has been possible to establish experimentally MIC toxicity. It is noteworthy, that methyl amine produced similar lesions, *albeit* less severe than MIC itself. DMU caused still milder and transient changes[14, 15].

Toxicological studies

The main purpose of toxicological studies was detection of the possible toxic substance(s) and to determine appropriate antidotes on an emergency basis. Thus the observation of the cherry red colour of lung and viscera assumed great importance, and even overshadowed all other factors.

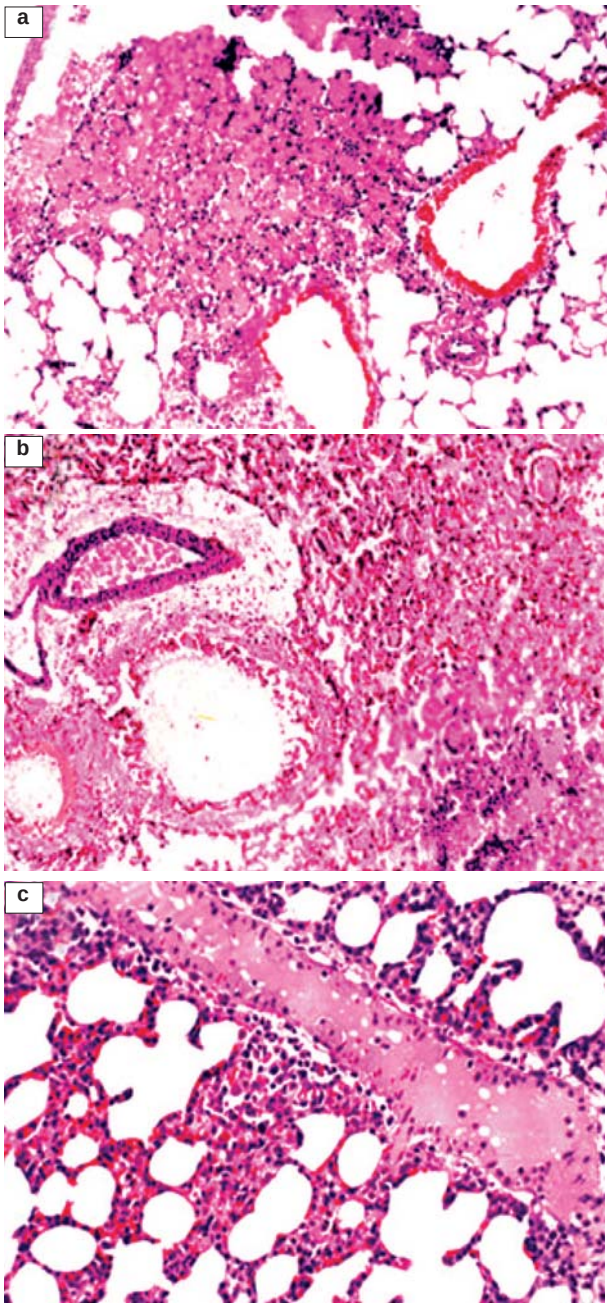

Figure 9 *a*–*c*. Early stages of experimental studies in rats. ***a***, 24 h picture of lung exposed to one LC50 dose of MIC showing eosinophilic necrosis of bronchiolar epithelium and lobular distribution of intra alveolar oedema; ***b***, Similarly exposed to two LC50 doses of MIC showing more extensive pulmonary damage as in the above; ***c***, Exposed to one LD50 dose of MIC subcutaneously; still the lung is the target with marked interstitial pneumonitis.

Issue of cyanide toxicity

On the basis of autopsy findings, Heeresh Chandra postulated cyanide toxicity as a causative factor. Max Daunderer, a German toxicologist, who came to Bhopal in

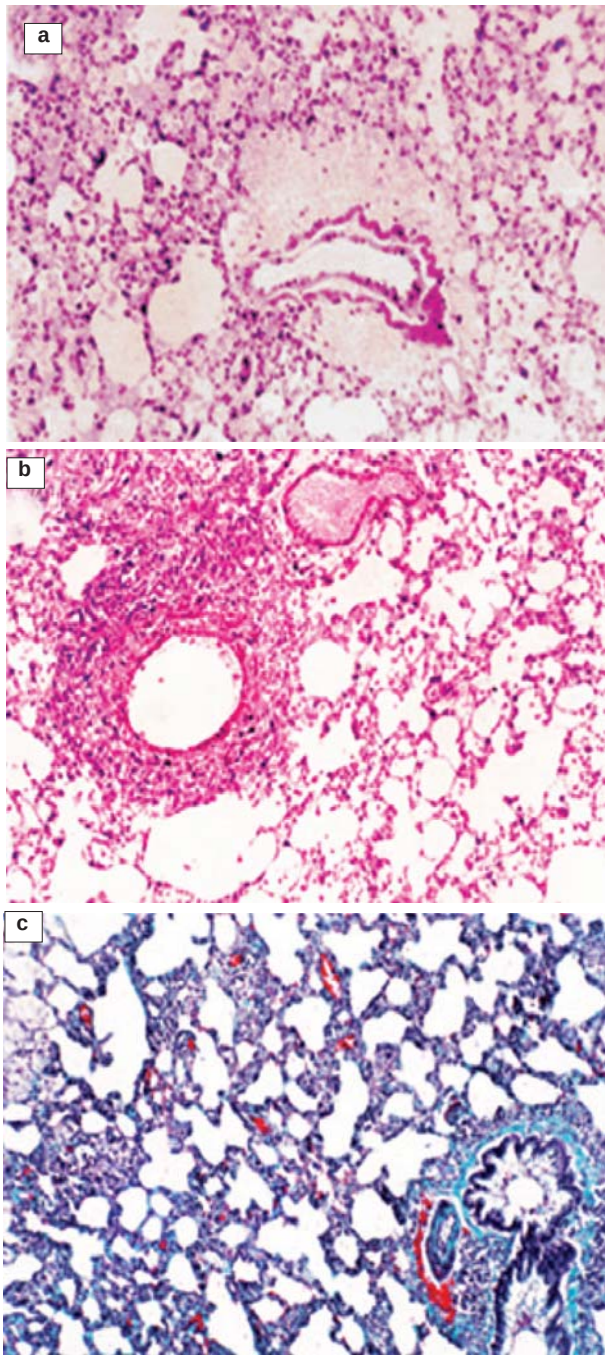

Figure 10 *a*–*c*. Late stages of experimental studies. ***a***, Photomicrograph of lung at the end of one week showing extensive pulmonary interstitial oedema; ***b***, Photomicrograph at the 4 week showing a picture of mild oedema, bronchiolitis and interstitial pneumonitis; ***c***, Photomicrograph at the end of 10 weeks showing a picture of diffuse pulmonary fibrosis.

the first week after the accident, also reiterated this fact. Based on his perception of possible cyanide toxicity, Daunderer brought diagnostic kits and ample supplies of the antidote, sodium thiosulphate. These developments spurred vigorous research into the possible causes of the 'cherry red' discolouration. A series of pathophysiological and toxicological studies were undertaken on an emergency basis. Certain preliminary investigations, with positive or negative results at each stage, paved the way for subsequent studies. 'Direct vision spectroscopy' and spectrophotometry of blood were initiated as early as 14.12.1984, i.e. second week after the disaster. However, none of the samples examined showed evidence of carboxy-haemoglobin or cyan-methaemoglobin. In all samples of all the victims, only the twin absorption bands characteristic of oxyhaemoglobin were seen.

Preliminary therapeutic trials with NaTS and enhanced urinary SCN response

Attempts to repeat the experiments of Daunderer met with variable results. Twenty very ill patients unrelieved after six weeks of adequate conventional treatment with bronchodilators, etc. were subjected to NaTS therapy. Twelve were fully cured but with residual tachycardia. Two had no relief while six were continued on treatment. However, in view of the reported dramatic response to injections of sodium thiosulphate, Sriramachari postulated

Table 3. First Double Blind Clinical Trial With NaTS. Urinary thiocyanate levels (in mg%) after injection of glucose or NaTS

Name	Resting	Post-glucose injection	% Change	Post NaTS injection	% Change
Sita	0.90	0.8	– 11	1.60	+ 100
Sakita	1.25	1.75	+ 40	2.80	+ 60
Azazuddin	1.00	1.35	+ 35	1.20	– 11
Satia Ban	0.50	0.30	– 40	3.00	+ 900
Devi Singh	1.50	0.80	– 46	1.20	+ 50
Sumet Lal	1.00	1.80	+ 80	0.80	– 55
Anna Malai	0.40	0.70	+ 75	1.60	+ 128
Rama Ji	1.40	0.60	– 57	1.40	+ 133
Prem Chand	2.00	0.60	– 70	0.70	+ 16
Zetru Nisa	1.80	0.80	– 55	1.20	+ 50
Mira Bai	0.50	1.20	+ 140	0.50	– 58
Raju Bagga	1.00	1.40	+ 40	1.60	+ 15
Kannaria	0.80	1.00	+ 20	1.70	+ 70
KS Pawara	0.80	1.00	+ 20	1.00	0
Beck Lal	0.70			3.15	+ 320
Puri	0.35			0.85	+ 157
Increase ³ 50%			3		10
Total cases			14		16

Fischer's exact test: $P = 0.031$.

Table 4. Follow-up study of urinary thiocyanate

Group	*n*	1985–86	1986–87	*t* value	Significance
A. Males – Below 40 years	44	Mean 1.2354	Mean 0.8931	4.5197	99.9%
		SD ± 0.5446	SD ± 4054		
B. Females – Below 40 years	41	Mean 1.2178	Mean 0.7066	5.4961	99.9%
		SD ± 0.5446	SD ± 0.3817		
C. Males – Above 40 years	36	Mean 1.27083	Mean 0.82297	3.4645	99%
		SD ± 0.7574	SD ± 0.3755		
D. Females – Above 40 years	12	Mean 1.0341	Mean 0.7166	2.0631	ns
		SD ± 0.4508	SD ± 0.2525		
E. Previous values of more than 1 mg	88	Mean 1.4538	Mean 0.8364	11.2143	99.99%
		SD ± 0.3760	SD ± 0.4040		
F. Previous value of less than 1 mg	45	Mean 0.66272	Mean 0.7134	0.1929	ns
		SD ± 0.2174	SD ± 0.3549		

the need to routinely monitor the urinary thiocyanate levels of the survivors. On the analogy of studies on smokers, it was considered worthwhile to monitor routinely survivors and exposees for urinary thiocyanate levels (NaSCN), both before and after the administration of NaTS. Surprisingly enough, they were elevated three to four times the normal. These findings supported a prima facie case of 'cyanide exposure'. In view of raging controversies prevailing at that time, abundant precautions were taken to ensure that the estimations were double-checked in some of the laboratories. Accordingly, the Institute of Nuclear Medicine and Allied Sciences (INMAS) was chosen. It was found that in comparison with the normals the affected population had twice or thrice-elevated levels, even after accounting for smoking habits, use of *Brassica* species of vegetables (cabbage and cauliflower). Several investigations on urinary thiocyanate were undertaken by the two toxicology projects.

Controlled double blind clinical trials

However, pitted against divided medical opinion, it was considered desirable to carry out controlled double blind clinical trials (CDBTs). With adequate precautions and statistical appraisal, it was established that NaTS was beneficial in relieving the symptomatology of the victims and was also accompanied by elevation of NaSCN (Table 3). This work was carried out by late Narayanan and Ramaiah, ably assisted by the local team of doctors, led by Abha Jain. The phenomenal improvement of the victims, including the relief of muscular weakness, was accompanied by further elevation of NaSCN. The CDBTs enabled a major policy decision of ICMR advocating widespread use of NaTS as a therapeutic measure.

Follow-up studies

Regular clinical trials on large cohorts of patients, both adults and children, were carried out for one and a half years. Serial observations on a large cohort of 300 patients clearly established the beneficial role of thiosulphate in the earlier period, in different categories of patients. The results are given in Table 4.

Monitoring of the urinary thiocyanate levels became an accepted norm. The total study involving 19,122 samples of urine including 1079 controls, added further confidence to the validity of the hypothesis of 'disturbance in cyanide metabolism'. Thus, one of the primary objectives of the toxicological studies was vindicated as early as February 1985. When declining trends were confirmed from the end of 1985, the treatment was tapered off. Out of 143 gas-exposed subjects studied in December 1984, 46.15% showed thiocyanate excretion of > 1.00 mg%. In the following years 1985–87, it came down to 41.05% out of 7752 subjects in 1985, 38.67% out of 5698 subjects in 1986 and 29.9% out of 4450 individuals in 1987.

Relapses and recurrences

However, therapeutic success of detoxification was not unqualified. A large proportion (nearly 30%) of patients had a 'clinical relapse' with increased pulse and respiratory rates, out of proportion to the degree of pulmonary damage or pulmonary function tests. Raised urinary NaSCN levels often accompanied such episodes. The patients responded well to repeated doses of NaTS, thereby confirming that the underlying changes were not merely subjective, but supported by objective scientific evidence.

Blood and tissue cyanide levels

There were initial hurdles in the matter of HCN estimations on blood and viscera with conventional methods. With the help of Head Space Analyser on a HPLC system, it was shown that HCN levels of the blood of victims or 'exposees', were more than double compared with living or normal autopsy samples (Table 5). Thus it was established that there was some degree of 'cyanide toxicity'.

Studies on Hb, 2–3 DPG and blood gases

The possibility of 'cyanate' being directly responsible for the cherry red discolouration was simultaneously pursued in other directions. Patients admitted to the 30-bed J.L.N. Hospital showed 'recurrent respiratory problems', due to excessive muscle weakness. Surprisingly, their pulmonary status and functions were reasonably satisfactory. But a majority of them exhibited abnormal changes in certain parameters of the blood such as Hb levels. The residents of J.J. Colonies seem to have consistently elevated Hb, which persisted for nearly 1–1½ years (Table 6).

Many of the victims also had abnormally high 2–3 DPG levels which were more than double the normal, of 1.0 ¼ mole/ml, as if they had been at a high altitude of 14,000 ft, for over two–three weeks. It took nearly a year for 2–3 DPG levels to return to normal.

Table 5. Blood cyanide levels (mg%) in controls and exposees

Group		*n*	Range	Average
Control	Live	15	5–30	20
	Post-mortem	31	10–50	25
Gas exposed	Live	34	50–110	70
	Post-mortem	43	60–360	150

Even after two months of preservation, autopsy samples (one sample from each group) on re-examination revealed no deviation from initial values.

Table 6. Haemoglobin levels of 14 g and above in the victims

Time of examination	*n*	Percentage
February 1985	8/20	40
June 1985	17/38	44
September 1985	49/120	37.5
March 1987	45/121	23.6

Table 7. Therapeutic reversal after sodium thiosulphate

Name	Status	PvO2	PvCO2
Azazuddin	Pre	25.4	47.3
	Post	34.0	53.5
Wahid	Pre	35.0	44.8
	Post	28.6	51.1

In view of the abnormal levels of Hb and 2–3 DPG levels, it was considered worthwhile to investigate the 'gas carriage and utilization mechanisms' of the victims. Further, it was also decided to study these parameters, before and after administration of NaTS and correlate the changes with the clinical improvement and alterations in the blood gases. Blood gas studies of arterial and venous oxygen and carbon dioxide were undertaken with ABL-3 Blood Gas Analyser and Oxymeter. Out of 26 patients, 14 patients had a PaO2 less than 85 mm of mercury (range 47.3 to 85.6 mm). Out of a batch of 14 untreated patients exposed to MIC, eight had PaCO2 below 35 mm mercury, which is the accepted lower limit of PaCO2. Out of the four treated patients, one had PaCO2 of 35.6 mm and all the rest had between 40 and 45 mm.

On the venous side, peripheral samples from the antecubital vein, only two out of the 23 patients investigated, had Hb less than 10 g%. Others ranged from 10 to 16 g%. Three out of 17 patients investigated had venous PvCO2 of below 40 mm of mercury; while nine out of 14 had values above 46 mm of mercury of PvCO2, i.e. above the upper limit. It is concluded that the patients had low PaO2, low PaCO2 and low PvO2 values, and normal or high PvCO2 values at rest, which did not increase on exercise. Also, the values in general could not be correlated on Hb basis.

Apart from clinical improvement after NaTS treatment, subjective or objective, the blood gases showed the following changes: There was no significant change in PvO2 which continued to remain low, indicating that the Hb showed some alterations in the oxygen-carrying capacity. Patients treated with thiosulphate had PaO2 ranging from 85 mm Hg upward and O2 concentration of 96%. More importantly, there was a demonstrable increase in PvCO2 at rest, which was further increased after exercise. The 'peripheral as well as central' catheterized venous CO2 concentration was equally elevated. This increase in PvCO2 *pari passu*, with definite clinical improvement, suggests the relief from 'chronic recurrent cyanide poisoning'. Typical cases showing increase in the PvCO2 values after NaTS therapy are shown in Table 7.

Studies on carbamoylation

The issue of alterations in the oxygen-carrying capacity led to further investigations on possible carbamoylation of the alpha and beta chains of the haemoglobin molecule. Ever since the occurrence of the Bhopal episode and the description of the cherry red discolouration of blood and lungs by Heeresh Chandra, several alternative hypotheses were entertained, even within the ICMR Toxicology Project. Towards the end of December 1984 itself, Sriramachari postulated that there could be several factors that can cause cherry red discolouration. Although the role of carbon monoxide and cyanide is well known, there are other lesser-known factors, such as aliphatic and aromatic nitriles and organic thiocyanates contributing towards the 'cyanogen pool'. Isocyanate itself, directly by carbamoylation of the end-terminal amino acids of the alpha, and beta chains of the Hb molecule can modify the oxygen affinity of blood. This could result in 'left shift of the Bohr effect'. Failure of deoxygenation or inability to shed oxygen due to reduction of CO2-carrying capacity could be the possible cause. The work of Cohen and Oppenheimer[16] supports such possibilities. These aspects were verified from January 1985 onwards.

Reduction of free amino groups. Reduction in the 'freeamino groups' of the samples of blood from Bhopal was assessed. It was soon established that there was a 20– 40% reduction of 'free amino groups', by the tri-nitro benzene sulphonic acid, TNBS Technique (Ramaiah *et al.*, unpublished data). It was not certain whether all the reduction was due to N-carbamoylation, since its greater interaction with SH groups of glutathione etc. could not be excluded[17].

Carbamoylation of Hb. Further investigations confirmed the presence of 'carbamoylated end-terminal valine residues' of the Hb molecules, both in experimental and clinical material from Bhopal[18]. Out of a batch of 60 samples of blood, 19 were found positive for N-carbamoylated methyl valine hydantoin (HVH)[19]. The scope of this work was enlarged at MLI, Bhopal, with the acquisition of HPLC and GCMS equipment[20]. Figure 11 shows the possible stages in the interaction of MIC with end-terminal valine residues of Hb chains. Mass spectro-

metric evidence of Ncarbamoylated valine agreed with Finnegan Matt Spectrometer Library Reference data (Figure 12). Increasingly, a large number of cases were shown to be positive for MVH[19] and occasionally 'non-methylated valine hydantoin' (VH)[21], suggesting the possibilities of break-up of MIC into HNCO, either in the atmosphere or demethylation processes in the human victims. Indeed, carbamoylation of Hb was proof of the MIC crossing the 'alveolar-capillary barrier' and spreading throughout the body, contrary to the initial statements of the Union Carbide from December 1984 onwards.

Tissue carbamoylation. Due to non-availability of proper reference standards, Heeresh Chandra and colleagues prepared a set of N-methyl carbamoylated hydantoins. First, the five amino acids, glycine, valine, phenyl alanine, methionine and threonine were used. Later, another batch of five amino acids, viz. serine, glutamic acid, glutamine, alanine and histidine were included. Unfortunately, the results with cysteine were not satisfactory. All the hydantoins were counter-checked with mass spectrometric data available with reference library. Chloroform extracts of preserved samples of autopsy tissues from exposees as well as controls, were screened for the presence of N-carbamoylated end-terminal amino acids of tissue proteins. A summary of the studies is given in Figure 13. Perhaps in the history of

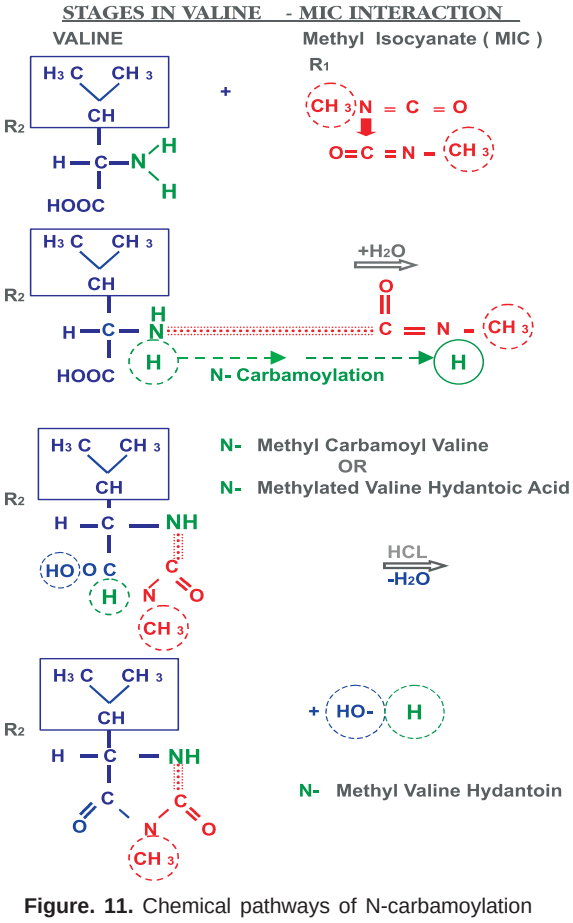

Figure. 11. Chemical pathways of N-carbamoylation

chemical disasters these findings are a unique example of tracing the offending chemicals to the blood and tissues of the victims.

Limitation of N-carbamoylation. However, the results of N-carbamoylation did not fully answer the issues of recurrence and repeated episodes of illness in the patients. It was observed that, on the analogy of some information generated in the ITRC, there was a reduction in the glutathione content of the blood[22]. The ICMR Toxicology Group felt that it could be due to transport across the tissues of the much faster and reversible S-carbamoylated glutathione.

Possible role of S-carbamoylation. Unfortunately, due to the lack of access to appropriate CI (Chemical Ionisation), GCMS, the problem could not be pursued further. However, Bailie and Slatter[23] had demonstrated experimentally that glutathione undergoes S-carbamoylation and functions as an exchange pool or a reservoir of the isocyanate in the body. For want of further evidence, perhaps it can only be surmised that reversible S-carbamoylation of acetyl choline esterase and aldolase may account for repeated episodes of muscle weakness, experienced by the Bhopal victims. As pointed by Heinrickson[24], the enzyme rhodanese can be selectively inactivated by phenyl glyoxal, combined with cyanide, at any of the two-cysteinyl residues at 247, 254 and 263 positions in the 'catalytic apparatus' of its B-domain. It is possible that in the Bhopal victims S-carbamoylated rhodanese is continually and reversibly inactivated under conditions of *in vivo* build-up of endogenous cyanide in the body. In all probability, the metabolic block of the 'catalytic apparatus' is corrected by repeated exposure to 'sulphane–sulphur' of NaTS. It is hoped that the dilemma will be resolved about 'recurrent endogenous chronic cyanide toxicity' following exposure to MIC. However it must be admitted that in our studies, as of then, we failed to demonstrate S-carbamoylation.

Tank residue studies

Attention has already been drawn to the reports on the generation of a large array of chemicals in the course of pyrolysis of MIC in Bhopal. The ICMR Toxicology programme confirmed and enlarged the findings. Mass spectrometric analysis of chloroform residue confirmed and enlarged the range of chemicals that could be detected[25] (Figure 14 *a*, *b*). Apart from the presence of known chemicals like MIC trimers, some of the unidentified compounds could be demonstrated in the autopsy samples of blood and tissues[26,27]. Although the toxic effects of such compounds remain an enigma, they are certainly of forensic evidentiary value.

Discussion

The moment the Bhopal gas disaster took place, the Union Carbide Company adopted a policy of *suppressio vary and suggestio falsi*. Concerted efforts were made to spread the message of disinformation. Invoking the rapid clearing of ocular changes, it was suggested that, on contact with aqueous surfaces, MIC is rapidly broken down to the relatively innocuous methyl amine and that MIC as such does not cross the alveolar-capillary barrier. No cogni sance was taken about the high reactivity and chemical binding of MIC to end-terminal amino acids of blood and tissue proteins as discussed by Cohen and Oppenheimer[16].

Secondly, it was suggested that acute anoxia and pulmonary oedema are transient and they would soon be corrected and the fluid reabsorbed. Strangely enough, ignoring the adverse effects of a highly reactive chemical, even Indian scientists echoed similar sentiments. In his Presidential oration of the January 1985 Session of the Indian Science Congress, Paintal[28] suggested that the oedema would disappear by itself, on the analogy of High Altitude Pulmonary Oedema (HAPO).

Thirdly, the issue of 'cyanide toxicity' was hotly contested[29]. The Union Carbide mobilized international scientific opinion to belittle the ongoing toxicological

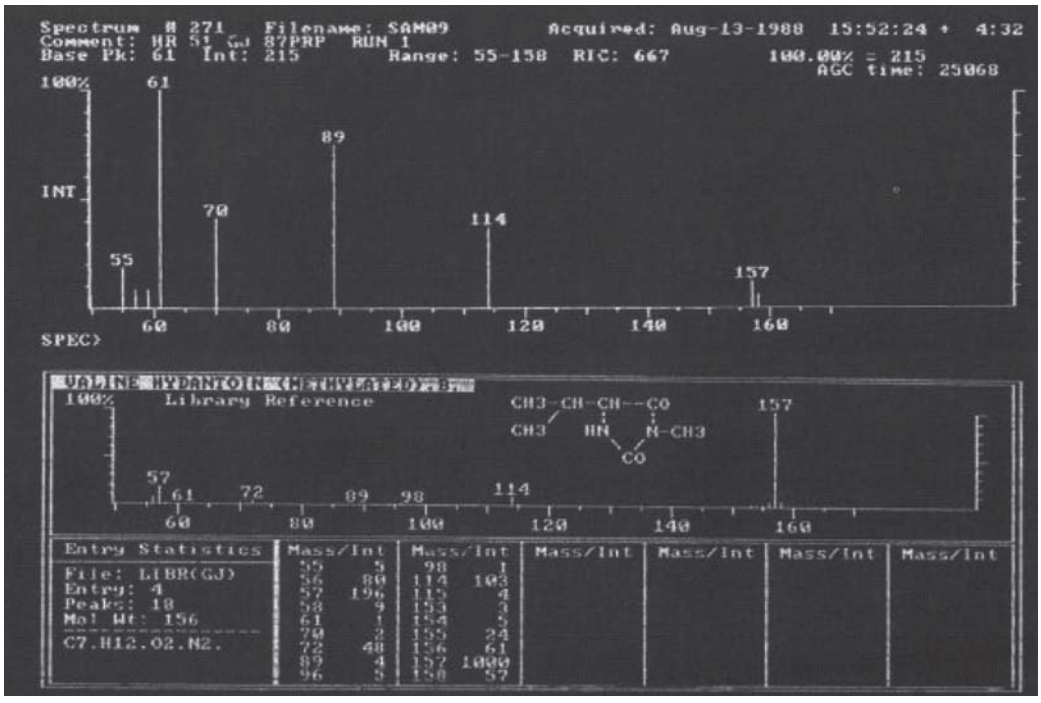

Figure 12. Comparison of mass spectrometric data of N-carbamoylation of valine residues of Hb of a victim compared with that of library reference standard.

Studies on carbamoylation

Source	1984		1985		1986		1987		1988		Total
	C	E	C	E	C	E	C	E	C	E	
Postmortem Blood	nill	7 (7)	nil	9 (4)	2 -	5 (1)	6 -	8 -	1 -	8 -	T-46 C-09, E-37
**	-	7/7+	-	2/2+	2/0	3/1+	6/0	3/0	2/0	2/0	T-27 C-10, E-17
Clinical	nil	nil	nil	3 (1)	2 -	3 -	9 -	13 -	1 -	2 -	T-33 C-12, E-21
Postmortem	nil -	35 (17)	1 -	18 (4)	nil -	14 -	16 -	41 -	12 -	54 -	T-191 C-29, E-162
**	-	13/7+	1/0	6/2+	2/0	2/0	3/0	2/0	2/0	1/0	T-31 C-08, E-23

In parenthesis are the number of samples showing chromatographic evidence of carbamoylation with reference to five amino acids; **Samples confirmed on GC-ITD; T, Total; C, Control; E, Exposed.

Figure 13. Summary table of N-carbamoylation of clinical and autopsy samples of blood and tissues.

studies in the wake of the Bhopal disaster (Ballantyne 1995, pers. commun.). The rapid binding or adduct formation of HCN with MIC was ignored or missed by both Indian and foreign media and the scientific community. It is indeed a matter of satisfaction that in the teeth of opposition, the toxicology projects by ICMR were able to generate viable evidence on some of these issues.

In the projects relating to toxicology, an attempt was made to describe the series of pathological and biochemical alterations. These studies were by no means exhaustive. In fact there are a few other studies which dealt with different aspects of clinical and laboratory studies, including immunological and chromosomal aspects[30].

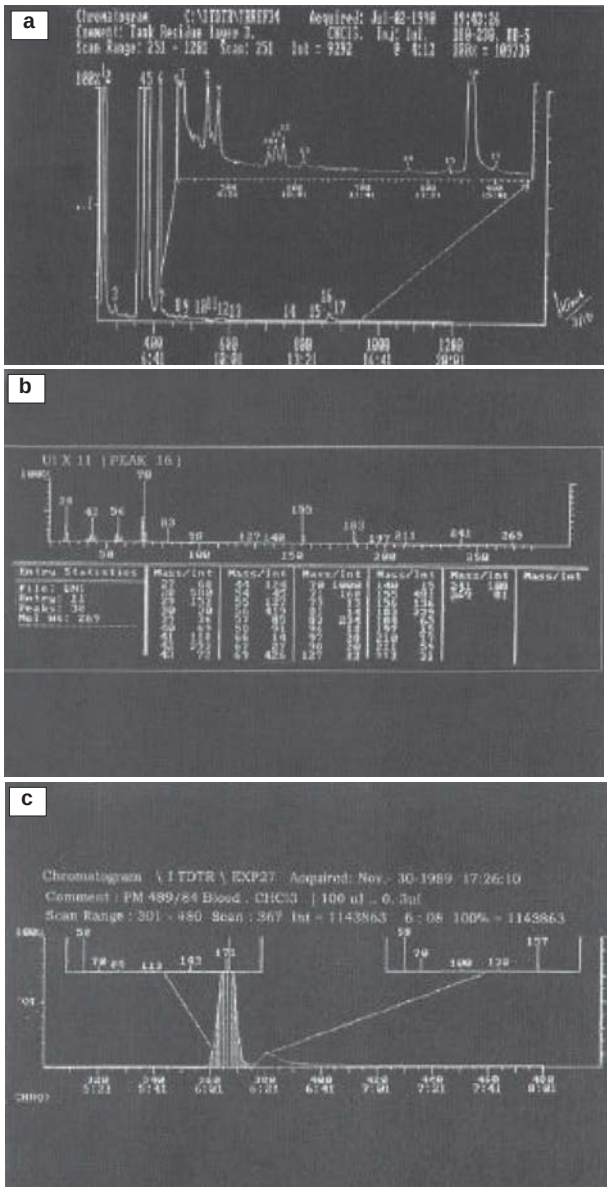

Figure 14 *a–c*. Mass spectrometric analysis. ***a***, Total ion chromatogram of chloroform-extract of the residue from MIC-Tank E-610; ***b***, Chromatogram and mass spectrum of unidentified compound, U.I. x 11, Corresponding to Peak-16 of the above; ***c***, Ion chromatogram with MS analysis of two unidentified compounds detected in the blood sample from PM 489/84.

However, it is not clear to what extent some of them are incidental changes unrelated to the pathogenesis of the lesions. Therefore in our studies an attempt was made to interpret the significance of the changes, which have a bearing on the patho-physiology or toxicity of MIC or its breakdown products.

As stated earlier, the Bhopal disaster cannot be attributed to exposure to a single chemical like MIC, at normal temperature. Instead, it is the combined effect of 'cold MIC' super-added with its pyrolytic products, following the powerful exothermic reaction in a closed stainless container or tank. Indeed, the complex and variegated clinical manifestations can be explained by correlating the adverse effects of several compounds (MIC, etc.). MIC toxicity in human beings in Bhopal can be broadly classified into two categories, viz. MIC itself and 'products of its pyrolysis'. It is impossible to distinguish the overlapping time sequences of each one of the components and in terms of any logical sequence of events. Within these limitations an attempt was made to interpret the findings.

Effects of pyrolysed MIC

Products of pyrolysed MIC, like carbon monoxide, ammonia and HCN could result in formation carboxy-haemoglobin, methaemoglobin and cyan-methaemoglobin. Theoretically, the last two can contribute towards the cherry red discolouration of blood and viscera. However, in the light of the negative spectroscopic findings, they do not seem to be present in significant amounts. But the permanent cherry red colour could be the result of damage of cytochrome oxidase system and damage to the respiratory centres, causing instantaneous death of many victims. The subsequent alterations in the blood gases such as fairly high PaO_2 but low $PaCO_2$ and high PvO_2 and low $PvCO_2$ resulting in cherry red colour, are strongly suggestive of 'acute cyanide toxicity'. Increased plasma and urinary thiocyanate and prompt response to NaTS gave further credence to acute cyanide toxicity. These findings were initially challenged as speculative. The elevated levels of blood and tissue cyanide in the Bhopal exposees provided further confirmatory evidence. Bhattacharya *et al.*[31] demonstrated that MIC pyrolysed at high temperatures caused inhibition of 'cytochrome oxidase activity' of brain tissues.

However, the subsequent features of reversibility and recurrence do not support the cyanide hypothesis. Also, the elevated Hb and 2-3 DPG levels need to be explained, apart from the cyanide issue.

Direct effects of MIC

The sudden physiological changes, following the exothermic reaction of MIC on the cellular and vascular components, can be attributed only to MIC. The flooding of the respiratory tract and 'massive pulmonary oedema' are perhaps responsible for acute anoxic anoxia, rapid stimulation of the 'respiratory centres', cerebral oedema and subarachnoid haemorrhage and eventual death. While these features by themselves, cannot explain the cherry red colour, the changes in blood gases, such as lowered PO_2, lowered arterial and venous O_2 and CO_2 levels can readily explain the increase in 2-3 DPG levels and increase of Hb levels and 'compensatory erythropoesis'. The experimental study following a single exposure to MIC, MA or DMU, also confirms the sequence of transudative and exudative changes demonstrated in the experimental studies closely simulating the human autopsy findings.

The available information about the high reactivity and binding of MIC end-terminal amino acids of blood and tissue proteins was virtually ignored. However, the uniqueness of MIC toxicity became apparent with the rapid development of chemical binding of MIC to end-terminal amino and SH groups. The successful demonstration of N-carbamoylation of blood within the first 120 day lifecycle, clearly provided an alternate explanation for the cherry red discolouration, elevation of Hb and 2–3 DPG levels as well as the characteristic alterations in the blood gases, especially the lowering of $PvCO_2$ values. The subsequent restoration of the latter, both in the peripheral and central venous pool, provides an explanation for the dynamic changes in the blood gases. However, the clinical phenomenon of recurrence and reversibility by NaTS provided a clue to the much faster S-carbamoylation, rather than recurrent exposure to cyanide. In all probability, this is due to rapid reversible S-carbamoylation of sulphydryl groups of key enzymes like rhodanese, esterase, etc. None of these features could be explained by mere accumulation of cyanide. It would appear that S-carbamoylation is probably responsible for the secondary chronic cyanide toxicity due to accumulation of endogenous metabolic products. It would appear that S-carbamoylation effects a 'labile' circulating component like glutathione and 'fixed' sulphydryl-specific functional enzymes. It is only when the reservoirs of S-carbamoylated MIC are exhausted, the secondary cyanide toxicity in general, and MIC toxicity in particular, is corrected. These mechanisms seem to be accelerated by periodic exposure to 'sulphane sulphur' in the form of NaTS. The effects of exposure to pyrolysed MIC in Bhopal appear to be a scientific challenge, which needs further elucidation.

Conclusions

The two ICMR projects on histopathology and toxicology have more than fulfilled the initial hopes and expectations. The sequence of pathological changes in the acute, sub-acute and chronic stages have been clearly delineated. Structurally, continuing respiratory impairment in a proportion of cases resulted in progressive pulmonary damage, including desquamative pneumonitis or fibrosing alveolitis or its variants, like DIPF and GIPF. Experimental studies with MIC and its aqueous derivatives have confirmed the pathogenesis and pulmonary changes after 'single exposure' are comparable to human autopsy findings.

The possibility cannot be excluded, at least initially, that some degree of 'exogenous HCN', due to thermal decomposition of MIC might have contributed to some degree of 'acute cyanide toxicity' and irreversible damage to cardio-respiratory and medullary centres of the victims. Perhaps in the survivors, the 'threshold limit values' were not reached, and thereby death was avoided. However, there was no escape from severe pulmonary oedema and extreme muscular weakness.

The 'cherry red discolouration' could be the immediate effect of HCN. In the long run, it would appear that Ncarbamoylation, which persists through the 120-day life cycle of erythrocytes, accounted for a greater part of the phenomenon. In Bhopal the question of repeated exposure to HCN is extremely unlikely, but 'endogenous liberation of cyanide' due to partial impairment of rhodenese and S-transferases seems plausible. This may account for the raised or elevated respiratory pulse rates and muscle weakness and protracted excretion of urinary thiocyanate.

While N-carbamoylation cannot be undone, it would appear that sulphydryl radicals contained in acetyl-choline esterase (ACE), aldolase and especially rhodanese are periodically reactivated and 'chronic cyanide metabolism' corrected. Normalcy is attained only when the MIC stored in the body is fully depleted. But, in the exigencies of an alarming human disaster, it has not been possible to try other potent sulphane donors described by Cohen and Oppenheimer[16]. It seems that the 'Biochemical Lesion' of Bhopal disaster may lie between the interplay of N- and S-carbamoylation.

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ACKNOWLEDGEMENTS. This article is dedicated to the memory of the several thousands of people who perished in the aftermath of the Bhopal gas disaster and the survivors who continue to remain crippled for the remaining part of their lives. My special tribute to the following departed souls who had contributed largely to our joint research endeavours in Bhopal: Prof. P. S. Narayanan, Prof. Heeresh Chandra, Dr M. P. Dwivedi, Dr C. R. Ramachandran, Prof. V. Ramalingaswami and Dr C. R. Krishnamurthi.

Starting my research career as a 'Lady Tata Memorial Scholar' 50 years ago, I deem it as fortuitous to be associated with the symposium in honour of Sir Dorabji Tata at the Centre for Tropical Diseases. I thank Raghunath who has given me an opportunity to present a comprehensive scientific re-appraisal of the Environmental Disaster of Bhopal, long after the dust and din have settled down. My special thanks are due to Shri Pushp Raj, Ms Seema and Dr A. K. Jain of IOP for rendering all possible help in my attempts at restoration of the colour and clarity of the old illustrations.

More than a score of individual scientists had participated in the multi-disciplinary and multi-institutional ICMR projects on toxicological studies in Bhopal. Special thanks are due to Prof. N. K. Ganguly, Dr S. Varadarajan and Dr V. S. Arunachalam, for all help from the several establishments of DRDO.

Received 10 December 2003: accepted 6 February 2004

Indian J Med Res 90, December 1989, pp 407-414

Bronchoalveolar lavage study in victims of toxic gas leak at Bhopal

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Accepted September 4, 1989

Bronchoalveolar lavage using flexible fibreoptic bronchoscope was carried out in 50 patients 1-2½ yr after exposure to the 'toxic gas' at Bhopal. Thirty six patients in the analysis were categorised into 3 groups (*viz.*, mild, moderate and severe), depending upon the severity of exposure. There was an increase in cellularity in the lower respiratory tract (alveolitis) of the severely exposed patients (in both smokers and non-smokers), compared to normals ($P < 0.05$). The increase in cellularity in severely exposed non-smokers was due to abnormal accumulation of macrophages ($P < 0.01$), and in severely exposed smokers, to macrophages ($P < 0.01$) and neutrophils ($P < 0.05$). Mild and moderately exposed patients did not show significant change in cellularity in lower respiratory tract, compared to normal individuals ($P > 0.2$). There was a trend towards increasing cellularity, as the severity increased ($P < 0.0001$) and higher numbers of total cells were seen in severely exposed smokers, suggesting that smoking is a risk factor. It appears, therefore, that subjects severely exposed to the toxic gas at Bhopal may have a subclinical alveolitis characterised by accumulation and possibly activation of macrophages in the lower respiratory tract. Smokers, who were exposed to the gas had in addition, accumulation of neutrophils.

It may be recalled that leakage of over 40 tonnes of 'toxic' gas from an underground storage tank of a pesticide plant at Bhopal on 3rd December, 1984, resulted in the death of a large number of people due to acute pulmonary oedema and respiratory failure. The survivors had persisting respiratory symptoms and it was suspected that a proportion of these patients might develop interstitial lung disease. The suspicion was due to the fact that some of the patients had symptoms and radiological findings suggestive of interstitial lung disease, though physiological investigations did not show clear-cut evidence of interstitial disease. However, previous studies[1] had shown that normal results on tests of pulmonary function did not rule out alveolitis. It is widely accepted that alveolitis is the essential feature of the pathogenesis of chronic interstitial lung diseases and the alveolitis is responsible for the injury and fibrosis of the alveolar walls[2-5]. The technique of bronchoalveolar lavage[6] (BAL) had made it possible to understand the character and function of the effector cells in alveolitis. A study was therefore planned to evaluate the tracheo-bronchial tree and to obtain samples of inflammatory and immune effector

cells from the lower respiratory tract, using the technique of bronchoalveolar lavage.

Material & Methods

All studies were carried out under protocols approved by the Indian Council of Medical Research, New Delhi. The procedures to be performed were explained in the local language and the consent was obtained in each case. This study was done 1-2½ yr after exposure to the toxic gas.

Pre-lavage assessment and investigations : Pre-lavage assessment and investigations of each patient included detailed history, physical examination, a full-plate PA chest X-ray examination, a 12 lead electrocardiogram, total and differential leucocyte count in the peripheral blood and pulmonary function tests (using Transfer Test Model C, P. K. Morgan Pvt. Ltd., Chatham, UK). Patients with pre-existing lung diseases were excluded from the study.

Bronchoalveolar lavage : Bronchoscopy and bronchoalveolar lavages were carried out as an out-patient procedure at Hamidia Hospital, Bhopal, in 50 patients. Patients were premedicated intravenously with valium (5-10 mg) and intramuscularly with atropine (0.8 mg). Four per cent xylocaine spray was used to anaesthetise the oropharynx and 5 per cent dextrose was given intravenously during the procedure. The flexible fibreoptic bronchoscope with an inner diameter of 2.6 mm (Olympus BF type IT 10, S. no. 2511484) was used for bronchoscopy and bronchoalveolar lavage. Usually the transnasal passage was used, but occasionally the bronchoscope was passed transorally. The lavages were usually done from three subsegments *viz.*, right middle lobe, lingula and left lower lobe. The tip of the bronchoscope was wedged in a subsegment. BAL was performed with 300 ml sterile 0.9 per cent saline at room temperature. One hundred millilitres of sterile saline in five 20 ml aliquots were infused through the fibreoptic bronchoscope into each of the three lobes in the lower respiratory tract. After each aliquot was infused, the cells and lavage fluids were recovered by gentle suction using 50-100 mm water negative pressure with an usual clinical suction apparatus and collected in specimen traps (Chesebrough-Ponds Ltd., Ireland). The fluid obtained by lavage was pooled in a sterile plastic cup (Falcon-Plastics, Oxnard, CA). The bronchoscopy and bronchoalveolar lavage procedures were done under continuous cardiac monitoring. Supplemental oxygen was administered during and 1-2 h following lavage. All individuals were observed for 3-4 h after bronchoscopy. There were no complications during bronchoscopy and lavage.

Immediately after lavage, the fluid was filtered through three layers of sterile surgical gauze and the volume was measured accurately. Cells were evenly resuspended by repeated aspirations with a 10 ml pipette. An aliquot was removed for determinations of cell number and preparations of filters for determinations of differential cell count. Rest of the fluid was centrifuged and preserved for biochemical and immunological investigations. The cells recovered by lavage were counted on a haemocytometer, using the unconcentrated lavage fluid[7] and expressed as cells per 100 ml of recovered fluid.

The lavage cell differentials were determined using filtration method[7,8]. Nitrocellulose filters 25 mm in diameter with 5.0 μm pores (SMWP-025-00, Millipore Corporation, Bedford, MA) were pre-soaked in absolute alcohol for 5 sec and mounted above a paper pad (AP-10-025-00, Millipore Corp.),

in a 15 ml graduated funnel with a fritted glass base (XX-100-25-14, Millipore Corporation). The filter was then washed with 15 ml of 0.9 per cent sodium chloride. 2×10^5 lavage cells were added to the funnel and the filter was washed with 15 ml of absolute alcohol. The filters were then removed from the funnel apparatus, mounted onto 25×75 mm microscope slides using bell clips (Bell Products Co.).

Cells collected on filters were stained with haematoxylin and eosin. The filters mounted on glass slides were washed in tap water (5 min), and distilled water (1 min) and stained in Harris haematoxylin (30 sec). Filters were 'blued' by washing in lukewarm tap water for at least 20 min. After 'bluing', filters were dipped in 50 per cent ethanol (1 min), and 80 per cent ethanol (1 min), and counterstained in eosin (2.5 min). The filters were then dipped in 3 changes of absolute alcohol (1 min each), one change of 2-propanol (2 min) and three changes of xylene (1-2 min each), until they are transparent and mounted on 25×75 mm glass microscope slides under glass coverslips using permount. Using oil immersion of a microscope, alveolar macrophages, lymphocytes, neutrophils and eosinophils were identified and 400 cells were counted from each preparation for deriving the differentials. Bronchial epithelial cells counted were always less than 5 per cent.

Normal controls : Ideally, lavages should have been done in normal individuals not exposed to 'toxic' gas at Bhopal. However, lavages could not be done in normal people because of the difficulty in obtaining consent from them. In the present study, therefore, the results of lavage performed on 12 non-smoking individuals from Madras (Vijayan, V.K., unpublished data) were used for analysis. None of these subjects had respiratory symptoms or abnormal physical findings, and all had normal chest X-rays and normal pulmonary function tests. None of the subjects were on any medication. Our results in normal subjects in Madras are comparable to those reported from Delhi[9] and Western countries[10].

Classification of severity of exposure : As the degree of exposure to the gas may not be the same in all individuals, the patients were categorised into three groups depending upon the severity of exposure. The three categories were as follows :—

(*i*) Severe exposure—If one of the members of the family died due to the toxic gas exposure or the patient had severe ophthalmic and respiratory symptoms, requiring immediate medical help with assistance from others, the patient was classified as having severe exposure.

(*ii*) Moderate exposure—After exposure to the gas, if the patient developed respiratory symptoms and required immediate medical relief, the patient was classified as having moderate exposure.

(*iii*) Mild exposure—After exposure, if the patient developed respiratory symptoms, but did not seek immediate medical relief because of mild symptoms, the patient was classified as having mild exposure.

Analysis : Fourteen patients were excluded from analysis due to the following reasons *viz*., poor recovery of BAL fluid (6); acute respiratory infections (3); interstitial fibrosis (2); prior bronchogram (1); old pulmonary tuberculosis (1); and normal smoker (1). The two patients with interstitial fibrosis were excluded because one (20 yr female) admitted that she had respiratory disease

prior to exposure to the gas. The second patient (a 65 yr old female) was excluded because she attributed all her symptoms to gas exposure (unconvincing history) and showed pus in the tracho-bronchial tree during lavage procedure.

All data between groups were compared using the two tailed Students' 't'-test and also with Mann-Whitney U test, and the results were found to be similar. The trend Chi square test was applied to see whether the level of exposure had any trend effect on the number of cells.

Results

All patients (n=36) included in the analysis were males. Female patients were not willing to undergo the procedure. The mean age was 35.7±10.6 yr (range 18-60 yr). There were 6 patients with mild exposure, 5 with moderate exposure and 25 with severe exposure. Eight patients were smokers and all of them had severe exposure. All mildly exposed patients had normal chest X-rays and pulmonary function. Three of five moderately exposed patients had radiographic abnormality of 1/0 (ILO, 1980 classification[11]) and two had obstructive ventilatory defect. Radiographic abnormalities of 1/0 or 1/1 were observed in 18 of 25 severely exposed patients. Obstructive and restrictive ventilatory defects were observed in 8 patients each in severely exposed group. The total and differential leucocyte counts and electrocardiograms were within normal limits in all subjects studied.

The mean values of total and differential cell counts in mildly and moderately exposed patients were not significantly different ($P>0.2$) from Madras normals (Table I). Severely exposed patients had a significantly elevated ($P<0.05$) total cell count in the lower respiratory tract compared to Madras normals. This was true whether all individuals or only severely exposed non-smokers were considered. However, the proportion of different types of cells recovered was similar to that of normals in all groups ($P>0.2$, normal compared to all others).

Table I. Total and differential cell counts in lower respiratory tract

(Data are mean + SD)

Group	Total cells ($\times 10^6$/dl)	M %	L %	N %	E %
Normal (Madras) (n=12)	14.4 ±6.5	83.6 ±7.1	14.3 ±7.0	0.9 ±0.9	1.3 ±1.2
Mild exposure (n = 6)	10.7 ±3.2	86.8 ±5.4	12.3 ±4.9	0.8 ±0.98	0
Moderate exposure (n = 5)	20.6 ±4.3	92.6 ±3.7	6.6 ±3.4	0.6 ±0.4	0
Severe exposure (n=25)	39.2* ±24.2	87.0 ±9.4	10.7 ±8.7	1.7 ±2.4	0.5 ±1.6

M, macrophages; L, lymphocytes; N, neutrophils; E, eosinophils

*$P<0.05$ as compared to the normal group

The range of total cells ($\times 10^6$/dl) recovered from the lower respiratory tract was 5.5 to 13.8 in mildly exposed, 13.8 to 25.3 in moderately exposed, 15.3 to 71 in severely exposed (non-smokers) and 21 to 136 in severely exposed (smokers) patients. Eleven of 17 (64.7%) severely exposed (non-smokers) and 7 of 8 (87.5%) severely exposed (smokers) had more than 2-fold increase in cells in the lower respiratory tract, whereas only one of 11 (9%) mildly and moderately

exposed patients had more than 2-fold increase. With increasing severity of exposure, there was a tendency for a higher proportion of patients to have increasing cellularity in the lower respiratory tract and this trend was statistically significant ($P<0.0001$).

The absolute numbers of different types of cells (total cells times differential percentages) are given in Table II. Among the inflammatory and immune effector cells recovered from the lower respiratory tract, macrophages showed a significant rise in severely exposed patients, compared to normals ($P<0.01$). This was true whether all individuals or only non-smokers were considered. There was also a significant rise in neutrophils in severely exposed patients who continued to smoke tobacco ($P<0.05$).

Table II. Mean absolute values of inflammatory and immune effector cells

	Cells ($\times 10^6$/dl)			
	M	L	N	E
Normal (n = 12)	12.10 ±5.54	2.10 ±1.39	0.1 ±0.17	0.2 ±0.14
Mild exposure (n = 6)	9.38 ±3.12	1.33 ±0.47	0.07 ±0.08	0
Moderate exposure (n = 5)	19.08 ±4.09	1.38 ±0.89	0.16 ±0.11	0
Severe exposure (non-smokers) (n = 17)	29.69* ±14.47	3.93 ±3.20	0.56 ±0.82	0.19 ±0.47
Severe exposure (smokers) (n = 8)	46.58* ±37.48	2.49 ±2.17	0.50** ±0.38	0.04 ±0.11

P values, * <0.01; ** <0.05 as compared to normals

Discussion

In severely exposed patients (both smokers and non-smokers), there was a significant increase in inflammatory and immune effector cells in the lower respiratory tract. Accumulation of inflammatory cells in the alveolar structures is referred to as alveolitis[12] and the observation of increased cells in severely exposed patients suggest that a proportion of these patients had alveolitis. The increase in cellularity in severely exposed non-smokers was due to abnormal accumulation of macrophages, whereas in severely exposed smokers it was due to macrophages and neutrophils. An increase in macrophages and neutrophils in the lower respiratory tract of smokers has been described by earlier workers as well[10].

Activated macrophages had been shown to produce various mediators which can cause injury to and fibrosis of the lung parenchyma[13]. The observation of increased number of macrophages in the lower respiratory tract in non-smoking severely exposed patients, even 1 to 2½ yr after exposure to the gas, suggests that alveolitis in this group of patients may have a deleterious effect on lung parenchyma. Usually, two types of cells are involved in alveolitis, such as macrophage-lymphocytic alveolitis in sarcoidosis[14], macrophage-neutrophilic alveolitis in idiopathic interstitial fibrosis[15] and macrophage-eosinophilic alveolitis in Tropical Eosinophilia[16]. However, a predominant increase in macrophages had been described in silicosis[17] and respiratory bronchiolitis[18]. Soon after exposure to the toxic gas, an increase in neutrophils varying from 72 to 98 per cent in BAL had been reported[19]. The occurrence of increased neutrophils in the lower respiratory tract soon after exposure[19],

and the observation of increased macrophages 1-2½ yr after exposure in the present study may be similar to the observation in mouse lung exposed to short Crocidolite asbestos fibres in which there was a rapid elevation of polymorphonuclear leucocytes, followed by a decline in polymorphonuclear leucocytes and a significant increase in alveolar macrophages between 5 days and 8 weeks, though it produced only minimal lung injury and fibrosis[20]. Similar increase in macrophages in BAL were reported in other experimental studies[21,22].

It had been recently demonstrated in non-smokers with long-term occupational exposure to inorganic dusts and functional evidence of interstitial diseases (asbestosis, coal workers' pneumoconiosis and silicosis), that the inflammation in the lower respiratory tract was dominated by alveolar macrophages[23]. The single exposure of large amounts of the toxic gas in our subjects has also resulted in an exaggerated number of alveolar macrophages in the lower respiratory tract, especially in severely exposed patients. Though the occurrence of increased numbers of alveolar macrophages may be non-specific in character, the demonstration that activated alveolar macrophages in various occupational lung diseases are capable of spontaneously releasing exaggerated amounts of oxygen radicals (O_2^- and H_2O_2), fibronectin and alveolar macrophage derived growth factor[23] suggests that long-term follow up of toxic gas exposed patients is essential to know whether the expanded numbers of alveolar macrophages are 'activated' and if so, cause any injury to the lung parenchyma.

The increasing cellularity in the lower respiratory tract, as the severity of exposure increases and also the higher total cells in severely exposed smokers compared to non-smokers, suggest that smoking is a risk factor. It was also further observed that the severely exposed smokers had a significant increase of neutrophils in the lower respiratory tract. Recent studies[13,24] had shown that activated macrophages and neutrophils in the lower respiratory tract can release mediators which were toxic to the pulmonary tissue resulting in permanent destructive changes. In view of the possibility that a portion of the increased alveolar macrophages and/or neutrophils (and/or their mediators) may be in direct contact with small airway epithelium[25], and some of these patients had already obstructive ventilatory defect, these cells/mediators may further damage the epithelium, resulting in progressive chronic obstructive lung disease. Health education of the community to avoid any type of pollution such as smoking tobacco, domestic and environmental pollutions is essential to prevent the early development of chronic lung diseases in these patients. Similarly, the role of steroids in suppressing alveolitis in severely exposed patients has also to be critically evaluated.

Acknowledgment

The authors thank the Indian Council of Medical Research, New Delhi, for providing facilities to undertake this study. The support provided by Dr R. Prabhakar, Director, Tuberculosis Research Centre (TRC), Madras, is also gratefully acknowledged. Statistical analysis was done by Shri P. Venkatesan, TRC, Madras.

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Indian J Med Res 99, May 1994, pp 231-235

Acute histopathological changes induced by methyl isocyanate in lungs, liver, kidneys & spleen of rats

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Accepted March 1, 1994

Methyl isocyanate (MIC), inhaled or administered subcutaneously (sc) at lethal concentration/dose caused essentially similar histopathological changes in all the viscera except for the lungs. The observed congestion of the viscera, foci of hepatocellular necrosis with widening of Disse's spaces in the liver and tubulo-rhexis with degeneration in the kidneys are attributable mostly to the initial shock. In addition, the lungs revealed more distinct route specific patterns of histopathological lesions. Inhaled MIC caused acute eosinophilic necrosis of the bronchial epithelium and frank alveolar edema, while MIC administered sc led to prominent vascular endothelial damage and severe interstitial pneumonitis with normal bronchial epithelium. The differential loci of damage in the lungs may be attributed to the immediate contact surface available for interaction with MIC.

Key words Kidneys-liver-lungs-methyl isocyanate toxicity-rats-spleen-visceral pathology

Inhalation of methyl isocyanate (MIC), a sensory and pulmonary irritant, leads mainly to necrosis of the epithelial lining throughout the respiratory tract and results in pulmonary edema at higher concentrations[1-4]. Though MIC has been shown to enter the systemic circulation in its active form and to reach the brain, liver, lung and kidney as evidenced by carbamylation of tissue proteins in rats[5] and in human beings[6], the main emphasis of earlier studies on the toxicity of MIC was on respiratory and irritant effects and the histopathological changes in the upper respiratory tract and lungs. Not much information is available on the acute histopathological changes in other visceral organs. Earlier, we had reported that subcutaneously administered MIC caused a mild to moderate degree of congestion in all the viscera of rabbits during the early period after intoxication[7]. There was cloudy swelling and some degree of coagulative necrosis in the proximal convoluted tubules of kidneys and varying degrees of centrilobular degeneration and necrosis in livers. Whether the resultant acute histopathological changes were the direct effects of MIC or the residual effects of systemic toxicity of MIC, is not clear. A few of the observed changes could have resulted from the hypotensive shock and severe tissue hypoxia[7,8]. In the present study, we have investigated the acute histopathological changes induced by MIC in rats exposed to lethal concentration/dose both by inhalation and subcutaneous (sc) routes, since the possibility exists for the chemical to enter the biological system through respiratory and non-respiratory routes (skin, eyes and mouth) during any chemical accident. One of the main reasons for including sc route was to demonstrate MIC-induced histopathological changes by eliminating the influence of sensory and pulmonary irritant effects.

Material & Methods

Methyl isocyanate (99% purity) was synthesized

and characterized as described earlier[7]. For inhalation exposure, pure MIC was used to generate MIC vapors in a static exposure chamber of 21.5 litres capacity, maintained at 25±1°C, while it was dissolved in olive oil for injection. The details on the acute LC_{50} and LD_{50} determination for MIC are described elsewhere[9].

Twenty eight male Wistar rats bred in Defence Research and Development Establishment, Gwalior, weighing 140±20g and maintained on a standard diet were used throughout the experiments. All were fasted overnight prior to the experiment while water was allowed *ad-libitum*.

In the inhalation group (n=10), rats were exposed to lethal concentration of MIC (1 LC_{50} or 1080 mg/m^3 or 465 ppm, for 30 min) while for the respective control group (n=4), rats were exposed for the same duration without MIC. In case of nonrespiratory route of exposure, lethal dose of MIC (1 LD_{50} or 328.6 mg/kg) was administered to rats (n=10) sc while in the control group (n=4) only olive oil (vehicle) was administered.

Only the rats which survived were sacrificed by cervical dislocation at 24 h after MIC exposure and subjected to initial postmortem examination. A minimum of 4-5 surviving animals from each group were utilised for subsequent histopathological examination. The animals that died during the course of the experiment were discarded to eliminate autolysed specimens. The lungs, liver, kidneys and spleen were removed at autopsy, fixed in 10 per cent neutral buffered formalin. The tissues were subjected to paraffin embedding, sectioning and staining with haemotoxylin and eosin for examination under light microscope.

Results

Of the 10 rats each exposed to MIC by either route, 6 died in 1 LC_{50} group and 5 in 1 LD_{50} group. There were no deaths in either of the control groups. In general, rats exposed to MIC by either route appeared sick, showing signs and symptoms of acute respiratory distress syndrome (ARDS). The severity of gasping was more in the rats which inhaled MIC than those receiving sc administration. Animals of both MIC treated groups did not consume either feed or water till the end of the experiment. Among the visceral organs examined, lungs were affected maximally in both groups of rats exposed to MIC. All other organs showed a mild to moderate degree of congestion, the intensity was more in rats administered MIC sc compared to the inhalation group.

Lungs : In rats exposed to 1 LC_{50} MIC, the sections of the lung showed in places the lumen of the bronchioles filled with strands of fibrin and occasional leucocytes. The bronchiolar epithelium showed intense eosinophilic necrosis and peribronchial edema. There was acute congestion accompanied by severe interstitial and intra-alveolar edema in the lung parenchyma (Fig. 1). On the other hand, in the animals administered MIC sc, the conducting airways up to the terminal bronchioles were apparently normal. There was severe interstitial pneumonitis and compensatory emphysema with the distension of the alveolar ducts and alveoli (Fig. 2). The highly cellular interstitial compartment extended around the alveolar ducts and peribronchial tissues. In addition, a noteworthy lesion was the damage of the vascular endothelial cells which were swollen and basophilic, suggestive of irritant effects.

Livers : In the 1 LC_{50} MIC group, centrilobular areas showed pallor, pyknosis and fine vacuolar degeneration while livers showed moderately severe damage in rats administered MIC sc. Occasionally, there were foci of eosinophilic necrosis of hepatocytes along with widening of Disse's spaces (Fig. 3).

Kidney : The parenchyma showed essentially similar changes in rats exposed to MIC through either route except for the degree of congestion. A mild to moderate degree of congestion was observed in rats exposed to MIC vapors while in the other group, there was marked congestion. Kidney parenchyma revealed cloudy swelling, tubulo-rhexis and degeneration (Fig. 4).

Spleen : Moderate to severe congestion was seen in both the MIC treated groups (Fig. 5).

Discussion

Following the chemical accident that occurred at Bhopal, considerable progress has been made to understand the toxic effects of MIC. The experimental studies by several investigators have brought out

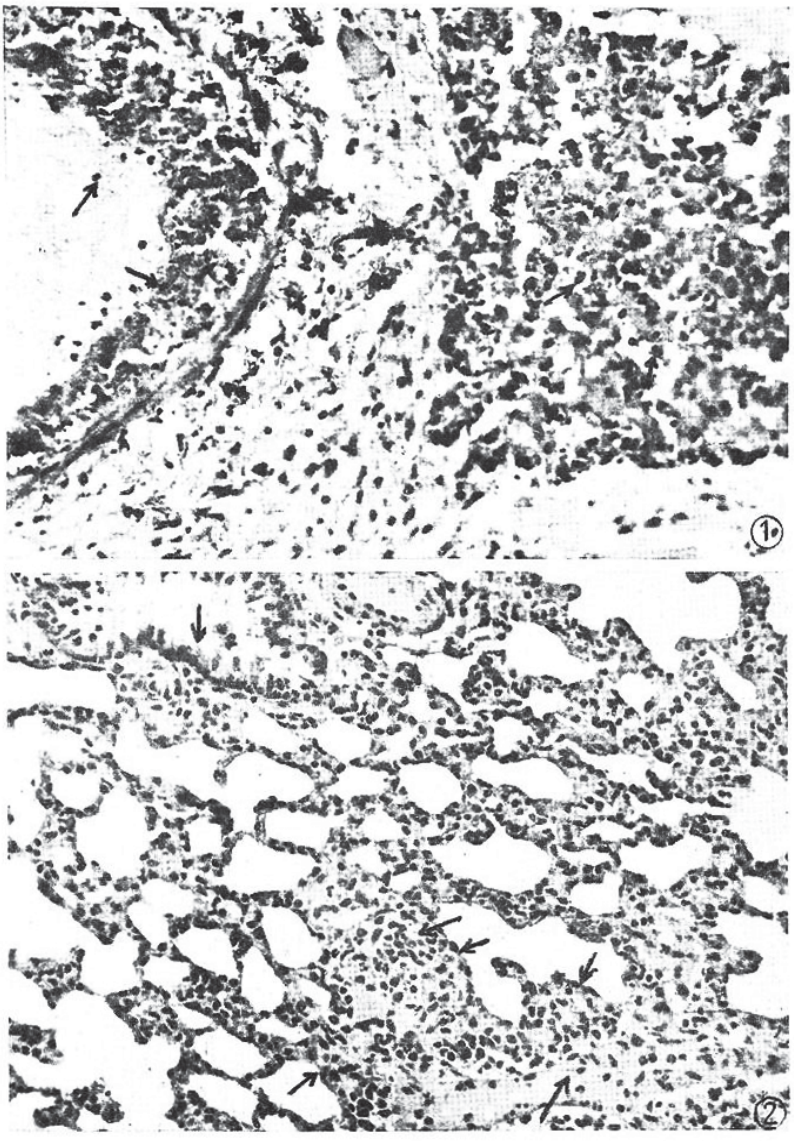
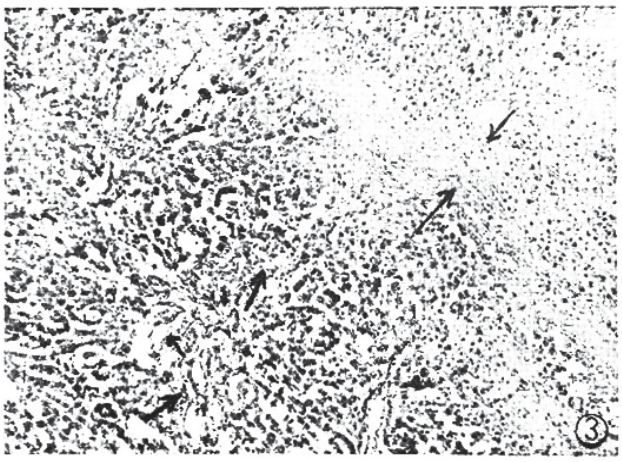
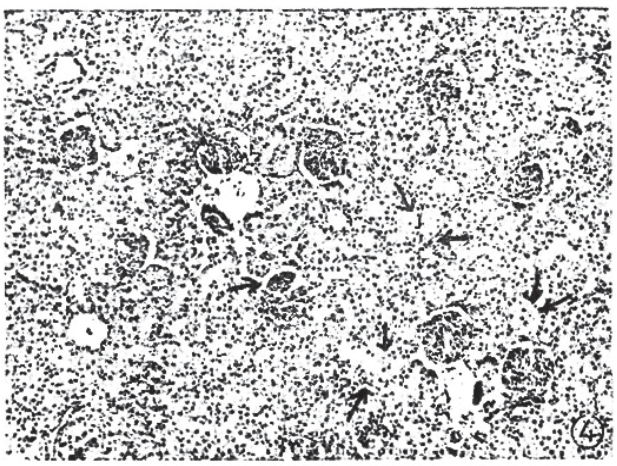


Fig. 1. Lung of rat exposed to 1 LC_{50} MIC shows the lumen of a bronchiole filled with strands of fibrin and occasional leucocytes, intense eosinophilic necrosis of bronchiolar epithelium with peribronchial edema, acute congestion accompanied by severe interstitial and intra-alveolar edema, H&E x 66 original. **Fig. 2.** Lung of rat administered 1 LD_{50} MIC sc shows apparently normal bronchial epithelium with no evidence of intra-alveolar edema. Instead, there is acute interstitial pneumonitis and the endothelial cells of the pulmonary vessels are swollen showing prominent reactive changes, H&E x 66, original.

the acute toxic effects of this highly reactive chemical to the respiratory system[1,2], the cardiovascular system[7,9] and at the subcellular level[10].

Most of the earlier studies on the histopathological changes of MIC exposure were found to be restricted to the upper respiratory tract with minimal effects on the lungs, possibly because of the lower concentrations used[2,3]. However, the results of the present study clearly demonstrate that the lung is the target organ of MIC toxicity irrespective of the route of exposure. Our results on the acute effects of inhaled





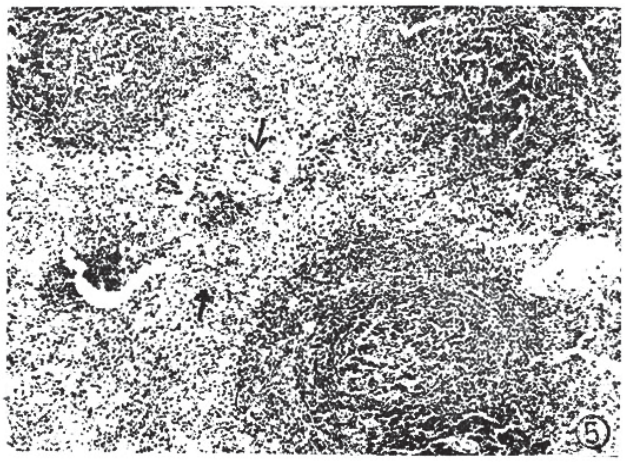


Fig. 3. Liver of rat administered MIC sc (1 LD_{50}) shows eosinophilic necrosis of hepatocytes along with widening of Disse's spaces. H&E x 33, original. **Fig. 4.** Kidney of rat inhaled MIC (1 LC_{50}) show congestion, cloudy swelling, tubulorhexis and degeneration. H&E x 33, original. **Fig. 5.** Spleen of rat exposed to MIC vapors (1 LC_{50}) shows moderate congestion. H&E x 33, original.

MIC on the respiratory bronchi, bronchioles and lung parenchyma are qualitatively similar to those described by Nemery *et al*[1] and to some extent with Pant *et al*[4], except for the severity of the lesions. The effective inhalation exposure concentration used by us (465 ppm for 30 min) is similar to that of Pant *et al*[4] but approximately two-fold that used by Nemery *et al*[1]. The histopathological changes such as congestion of the viscera, foci of hepatocellular necrosis with widening of Disse's spaces in liver and tubulorhexis with degeneration in kidneys are mostly the manifestations of the initial shock[11], although the direct effects of MIC cannot be ruled out. Similar histopathological changes characteristic of varying degrees of shock have been observed in the early human autopsy materials at Bhopal (unpublished data).

In addition to the manifestations due to shock, other histopathological lesions were observed in the lungs of rats exposed to MIC by the different routes. The differential loci of damage may be attributed to the immediate contact surface available for MIC interaction, the bronchial epithelium in the inhalation group and vascular endothelium in the other group. The necrotising effects of inhaled MIC may be attributable to its corrosive action. It is possible that the damage to the endothelial cells is a manifestation of lesser cytotoxic effects of MIC. Alternately, it is possible that it could be due to the hydrolytic derivatives of MIC such as methylamine. Further, in rats administered MIC subcutaneously, although the upper respiratory tract is spared, there was severe interestitial pneumonitis. The low hydrolytic stability of MIC suggests that these lesions could possibly be caused by methylamine, its hydrolytic derivative. Moreover, a recent report reveals that methylamine could cause mucous membrane and respiratory tract irritation leading to respiratory depression[12], and a

single exposure to a near-lethal concentration of it resulted in tracheitis, bronchitis, pneumonitis and pulmonary edema[13]. These preliminary findings are significant for unravelling the long-term sequelae of the two patterns of toxicity in relation to MIC and its aqueous decomposition products.

Acknowledgment

Authors are grateful to Dr R.V. Swamy, Director, Defence R&D Establishment, Gwalior, for his keen interest in this investigation.

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Experimental Studies on Single Exposure of MIC and it's Aqueous Derivatives

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IMMEDIATELY following the Bhopal Toxic Gas Disaster, Union Carbide, the manufacturers of methyl isocyanate (MIC), widely circulated available information about MIC. It was specifically reiterated that MIC, on contact with the aqueous surfaces of the respiratory tract, breaks down into relatively innocuous methylamine (MA) and/or dimethyl urea (DMU). It was suggested that, the survivors may suffer from 'transient pulmonary edema", but there may not be any no permanent damage.

Our extensive autopsy studies in Bhopal, immediately following the single massive exposure, revealed a spectrum of pathological changes in the lung, the target organ. There was little doubt that MIC caused a 'chemical burn' of the respiratory epithelium. The necrotizing bronchiolitis was variable and patchy. But pulmonary edema and interstitial pneumonitis were widespread. There were doubts whether the aqueous breakdown products of MIC contributed to the other two lesions. In either case, the issue of progressive or permanent damage, like pulmonary fibrosis, remained open.

Soon after Bhopal, several experimental studies were mounted worldwide on "Inhalational Toxicology of MIC". But for notable exceptions, very few had relevance to the emerging clinico-pathological scenario in the Bhopal victims. Either too high or too low dosages of MIC were employed. There were no sequential studies to ascertain the progression to pulmonary fibrosis. Instead of a "single exposure", they were often repeated, perhaps to study hyper-sensitization., Above all, the comparative effects of MIC and its aqueous derivatives, MA and DMU were not investigated.

To resolve some of the issues, we undertook an appropriate experimental study on rats, which were subjected to a single exposure of lethal or sub-lethal concentrations of pure non-pyrolysed MIC, MA & DMU. Two different routes, inhalation and subcutaneous injection (s.c.), were employed, to offset inherent dosage variations and the sensory pulmonary-irritant component in the former. The dosage of MIC was determined from the accurately weighed quantity of MIC, which evaporates within the confines of the 21.5-L exposure chamber. After 30 minutes 01 exposure by inhalation or s.c. Injection, followed by 48 hour observation period, a LC_{50} value of 19 µmol/I and a LD_{50} value of 5.75 mmol/kg of MIC were first established. The amounts of MIC required for the 3 scheduled dose levels of 0.5, 1.0, and 2.0 for both LC_{50}sand LO_{50}s were calculated. Due to high volatility of MIC, it was mixed with olive oil before s.c. administration. In addition, corresponding equimolar concentrations of MA were employed by both the routes. As DMU does not vaporize, only the s.c. route was tried. After a single exposure with each chemical at the three dosages, acute, short-term and long-term experiments were undertaken. It was planned to sacrifice at least four to five animals from each batch at stipulated periods of 24 hours, 1, 4,10 and 16 weeks. As many of the animals failed to survive at the higher dosages, the experiments were repeated with 0.75 LC_{50} and 0.75 LD_{50} dosages. Further, the duration was restricted to 10 weeks. All dead animals were ignored, to avoid the risk of autolytic changes. All animals that survived upto the stipulated periods were sacrificed and tissue blocks of the lungs were processed for detailed histopathology, including connective tissue changes.

Irrespective of the route of administration, the lung was found to be the target organ in all the groups. Within each modality of administering MIC and MA, there was a dose-dependent severity of response. In the acute phase, MIC by the inhalational route showed by far the most severe lesions. They included ex1ensive necrotizing bronchitis, severe intra-alveolar & interstitial pulmonary edema and interstitial pneumonitis. By contrast, when MIC given by the s,c, route, the necrotizing bronchial epithelial changes were totally absent. Intra-alveolar pulmonary edema was not evident. except with the higher dosage of 2.0 LC_{5o}. Interstitial pneumonitis was very conspicuous. However, a new, unique and consistent feature was endothelial damage of the pulmonary vascular endothelium. It was characterized by marked swelling and intense basophilic change, suggestive of hyper-reactivity of the endothelial cells. By contrast, the only note-worthy lesion produced by MA and OMU in the acute phase was a variable grade of interstitial pneumonitis.

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At the subsequent stages at 4 weeks, the necrotizing changes in the 'Inhalation Group' virtually disappeared with full restoration of the bronchial epithelium and occasion1 goblet cell activity. However. a moderate degree of pulmonary edema and marked interstitial pneumonitis persisted. In the 's.c. Group', on the other hand, the vascular endothelial damage persisted, accompanied by a dominant feature of a marked interstitial proliferative cellular reaction.

By the end 10 weeks, the necrotizing bronchial epithelial changes as well as the vascular endothelial damage disappeared. However, the two polar types of lesions seemed to converge towards interstitial pneumonitis and eventually, diffuse interstitial pulmonary fibrosis (DIPF).

Thus this investigation has established that single inhalational exposure of MIC or MA and DMU, have dose-dependent and differential effects on the respiratory system. Necrotizing bronchial epithelial changes and intra-alveolar edema are unique to MIC inhalation and are fully reversible. Interstitial pneumonitis caused by MIC or MA and DMU, is more permanent and progresses to pulmonary fibrosis which could be mediated by the final common pathway of metabolic transformation of all the three compounds to formaldehyde and formic acid, the well known stimulants of pulmonary fibrosis. The occurrence of endothelial damage of the pulmonary vasculature accompanied by massive interstitial proliferative cellular reaction caused by s.c. administration of MIC is a unique observation which needs to be pursued further. This may have relevance in the context of absorption of some Isocyanates through the skin during occupational exposures.

It would be important to study in future, the patterns of pulmonary damage including Immunological reactions caused by different Isocyanates and their derivatives.

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CHAPTER

5 PATHOPHYSIOLOGY & INHALATION TOXICOLOGY

On 3rd December, 1984, the very first day after the exodus of dead bodies to the mortuary, Prof. Heeresh Chandra postulated a tentative diagnosis that death was due to poisoning by irrespirable gases, resulting in **"Cytotoxic / Histotoxic Anoxia".** Initial, as well as subsequent, deaths only confirmed the 'Hypothesis of Hypoxia'. International opinion also veered around the same view, (vide Bucher 1987). BGD is unique, both from historical and scientific points of view. Lack of prior information and absence of coordinated scientific approaches contributed to considerable misunderstanding. The Toxicological Studies of ICMR enabled the gradual emergence of scientific rationale underlying multifactorial Pathophysiology of BGD.

Patho-physiological Issues in Bhopal

Impact of Environmental Factors: Even normally there are subtle problems related to 'physiological adjustment to variations in diurnal temperature, especially with the onset of winter in a place like of Bhopal. It is not often realized that there are limits to adjustment of the human body to 'diurnal variations', which are taken for granted. A gradual rise of an average of 10°C is believed to be physiologically comfortable. The adverse effects of the diurnal variations are normally managed by seasonal modifications of 'dress and ventilation' and avoiding sudden exposure to large variations. Similarly, the phenomenon of 'moist heat', without rise of temperature, makes the environment uncomfortable. It is also very well established that although the temperature of steam is only 100°C, the 'latent heat' that is generated can greatly damage the tissues that come in contact. The physiological response to extremes of diurnal variation will be virtually impossible for the body to adjust. It is more so, when it is not gradual but abrupt, and unanticipated. In terms of increased heat generation by MIC, some of its physical properties are worth brief mention. It boils at 39.1°C at 760 mm Hg. The heat of vaporization at 1 Atmosphere is about 223 Btu/lb and the heat of combustion at 25°C is 8177 Btu/lb. It also indicates that the time to acclimatize and take necessary precautions are virtually absent in the Bhopal Gas Disaster at midnight. Consequently, on 3rd December 1984 Bhopal witnessed an unusual phenomenon of apparent rise of day time temperature.

Perhaps one can explain the phenomenal rise in temperature as an 'exothermic reaction' of MIC with its affinity for water and atmospheric moisture. In fact the movement of the **AEROSOL** has been identified with the 'watery terrain' of the lakes. As per UCC manual (1976), DMU, TMU, and Mono-Methylamine are the 'end products' generated in such a reaction. The UCC's contention that liquid MIC, immediately on contact with water undergoes hydrolysis into the vapor or gaseous state is a wrong assumption. Instead, depending upon the temperature & pressure (Central Water & Air Pollution Control Board, 1985), the forgoing reactions could have taken place. Any delay could be called 'latent period of reaction', leading to formation of other compounds enumerated elsewhere (Table 8.5). Incidentally, the body tissues of the victims showed high concentrations of Trimer, and Dimer (Chandra et al, 1991; Saraf et al, 1995), but methylamine which simulates 'smell of rotten fish', was found only in one autopsy and that too, as late as 20th December, 1984.

Incidentally, a similar phenomenon of elevated temperature seems to have occurred on 16th December 1984; the rise of apparent temperature reached a maximum as felt in the afternoon. However, the scientific explanation propounded after the first event does not explain the subsequent event. The latter was a controlled activity in the Union Carbide Factory during the 'Operation Faith' when MIC was being converted to SEVIN. During this exercise, on all days of Operation Faith (Varadarajan, 1985), i.e. 16th to 22nd December, 1984, water was sprayed by helicopters to mop up any unforeseen local release of MIC from Tank 611; thereby some 'latent heat' might have been generated.

But these two independent events provoked the Toxicology team of ICMR to cogitate and speculate about the actual implications in the context of the BGD. The only escape could be found in the suggestion that the activated MIC

generated heat absorbable by the atmosphere at an altitude of 30 meters and the apparent change in the Environmental Temperature caused some transformation in its chemical activity. The possible explanation that could be advanced is that Latent Heat that was generated by the MIC vapor / gas, could be responsible. On the other hand, the bulk of the changes appear to have originated within Tank 610 and determined actually by the 'quantum of water' that had entered.

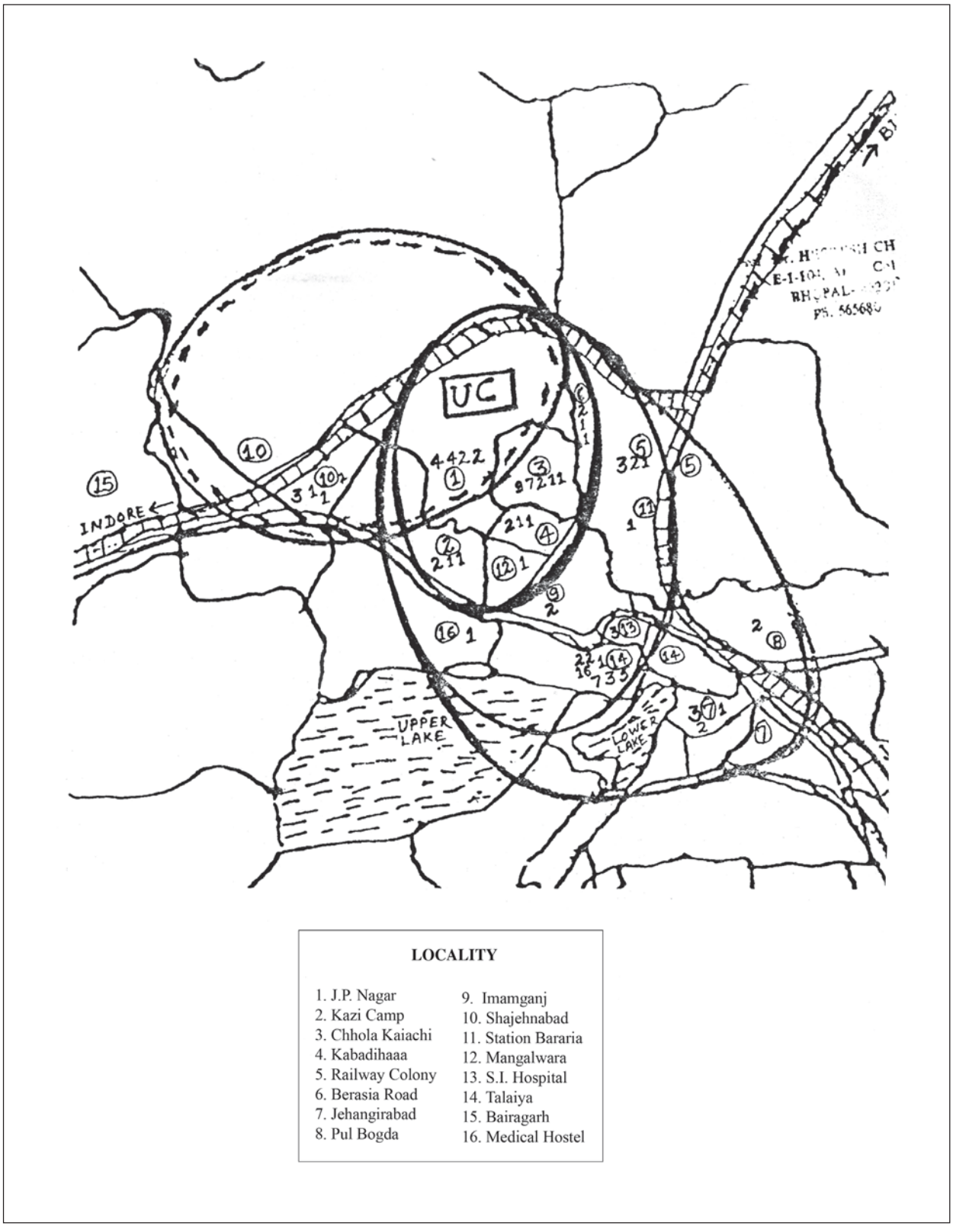

Figure 5.1: The Mortality-cum-Morbidity Map of BGD

It is also noteworthy that deaths and severity of exposure constituted the basis for outlining a map for subsequent epidemiological studies of the ICMR. This map (Figure 5.1) shows the Epicenter at Union Carbide factory and a parabolic field of exposure and also shows that the Aerosol first traveled West~North, as an elongated parabola with Union Carbide Factory as the Epicenter and later traveled South~North along-side the lake contours.

Since the temperature certainly would have gone beyond 218°C, which is the 'critical temperature of MIC' (UCC Manual, 1976), it is possible that the chemical properties of the original MIC component that came out of the Tank 610 (reportedly in the form of Gas), would have undergone alterations or degradation. By contrast, nearly 28 scientific publications in Environmental Health Perspective, Vol. 72, 1987, consisted of papers based on Experiments with fresh or cold MIC or its vapor, but not in the degraded 'Gaseous Form'.

The Experimental Studies at DRDE Gwalior: On the other hand, Dr. PK Ramachandran and his colleagues, especially, Dr. Vijayaraghavan & Dr. Shrivastava worked with pyrolysed as well as non-pyrolysed MIC. Strangely enough, although pyrolysis was not associated with any liberation of HCN, they found MIC interfered with 'Cyanide Metabolism' as shown by **'elevated blood SCN^- levels'**. However, they also found significantly increased Serum Thio-Cyanate Levels at the end of the 1st hour, though not at 4 hours. But unfortunately the issue of elevated Serum Thio-Cyanate, for longer periods of time, awaits re-confirmation. It is a very well known fact that MIC undergoes reaction with itself (CWAPCB, 1985; UCC Manual, 1976). What were altered properties of these ultimate compounds remains a mystery, since it cannot be simulated (Bucher, 1987). Surprisingly, there are no toxicological studies on human or animals of these available compounds to know the nature of damage, manner of assimilation, detoxification and excretion. If, any experiments have to be done at all, they have to be done at least within parameters in which main constituents of Bhopal Methyl Isocyanate were kept.

The impact of Blake and Ijadi-Maghsoodi (1982): In this context, it is worth recalling the pioneering provocative work of 1982, two years prior to BGD. It has brought to light additional toxicity of pyrolytic derivatives of MIC, viz., CO & HCN, etc. which particularly inhibit the **'cytochrome oxidase c'** system of ATP cycle (Stryer, 1975). They also alter the function of Hb through formation of Met-Hb and CO-Hb. In the annals of the Bhopal Gas disaster, the Toxicology Project of ICMR fully utilized the practical implications, especially the hidden indicators such as "Cold Trap". The MIC which is twice heavier than air, breaks down with progressively rising temperature and generates two basic lethal compounds viz., CO as well as HCN, along with a few other not-so-toxic constituents.

In addition, the seminal paper of 1982 also suggests that freshly generated HCN combines physically with MIC and forms an Adduct or a 'cold trap'. With the enhanced density of the latter, the Aerosol tends to come down (gravitate) and contribute to a 'divided or dual toxicity'. The **Initial Mortality** could be a result of liberation of HCN and the **'Long-Term or Delayed Morbidity'** due to the MIC component of the **Adduct**. Such a phenomenon of reactivity of MIC could have been responsible for its earlier use as a potential War Gas by US department of Defense (Chemical and Engineering News, 1984) and allegedly used in Iran-Iraq war in 1985.

Synergistic Effect of CO & HCN: As stated earlier, the thermal decomposition of MIC to CO & HCN have been demonstrated by Blake and Ijadi-Maghsoodi, and their role in BGD has been partially established by the ICMR studies of Bhopal. Perhaps the BGD illustrates the first known occurrence of the phenomena of thermolysis of MIC contributing to liberation of HCN causing massive early deaths and delayed cyanide toxicity. It would appear that similar mechanism might be operating in nitrogenous artillery chemicals used in warfare. Indeed, the extent and range of "Weapons Emissions" of noxious gases like CO & HCN have been studied under different operational conditions, with particular reference to their synergistic effects. Although, the nature of the 'Artillery Chemicals' used are not mentioned, the releases of the noxious gases and their effect on users/operators of gun & rocket devices have been recently studied. In a remarkable publication entitled "Combined Exposure to HCN and CO in Army Operations: Final Report" by the National Academy of Sciences USA, (National Research Council Report, 2008), the scientific basis of concurrent CO & HCN emissions at the operational site as well as the weapon handlers' (user personnel), and the need for their amelioration, protective treatment and therapeutics have also been envisaged (Halperin, 2008).

Dual Patterns of Lethality & Morbidity of Bhopal Gas Tragedy: The data, in retrospect, reveals two distinct phenomena. One is restricted to the **first three days** and is probably due to inhalation of free HCN or its liberation ***in vivo*** *from MIC~HCN adducts.* Distinct from 'Direct or Acute Cyanide Toxicity', the Sub-Acute or Chronic Type, appears to be due

to interference of 'Cyanide Metabolism', probably due to inactivation of rhodanese by S-Carbamoylation of one of its constituent triple -SH radicals. The prompt response to NaTS therapy is accompanied by increased urinary SCN^- excretion. This phenomenon of recurrent or chronic cyanide toxicity continued upto the end of the first quarter. Incidentally, the ITRC, Lucknow demonstrated consistent fall of Glutathione levels in Bhopal Gas Victims (Srivastava et al., 1988).

Due to unawareness of the underlying complex Biochemistry of these factors, the Scientific Community of India and abroad, as well as UCC and even some of the activists like Dr. Ramana Dhara (Gassert & Dhara, 2005), exploited the scientific confusion by decrying it as 'so-called Cyanide Toxicity' in Bhopal victims. The aspersion was promptly contradicted through 'Science Correspondence Columns of Current Science' by Sriramachari (2005).

Misinformation of UCC Dispelled: From the very beginning of the Bhopal saga, the UCC propagated a wrong idea that MIC does not cross the lung or capillary~alveolar barrier, belittling the need for detoxification. This was in spite of diagnosis given on the first day that extensive damage to internal organs had taken place due to carry-over of toxicological damage to vascular and respiratory systems. Later, many studies are available, which are in consonance with Bhopal observations from the very beginning (Bhattacharya et al., 1988; Ramachandran et al., 1988).

Toxicology Group of ICMR established early enough that impaired O_2 transport due to chemical impairment of Hb and damage of the Respiratory System seemed to be the cause of mortality (Rao et al., 1986; Rao et al., 1989; Sharma et. al., 1990; Sriramachari et al., 1991; Chandra et al., 1991 & 1994; Saraf et al., 1995). The experimental studies of UCC (1976) on guinea pigs indicated that the arterial tension of oxygen was reduced. Further, as postulated by Dr. Sriramachari in early 1985, specific amino acid residues like Valine of Hb molecules showed reduced O_2 binding capacities due to chemical modification in the victims. A marked reduction of the End-Terminal Amino Groups of Valine residue of Hb of Bhopal Exposees by 'TNBS studies' was demonstrated by Sriramachari, Ramaiah and Roman Reddy. It was soon confirmed by presence of N-Carbamoylated Valine residues (Bhattacharya et al., 1988; Ramachandran et al., 1988; Sriramachari et al, 1991). Almost simultaneously, Dr. Narayanan, Prof. Heeresh Chandra and Dr. Sriramachari reported elevated levels Hb & 2-3 DPG levels, simulating changes of an altitude of 14000 feet! In another series, their physiological implications, in terms of Blood Gas alterations were characterised by lowered PaO_2 & $PaCO_2$, lowered PvO_2 but elevated $PvCO_2$. The summary of the physiological changes were discussed in Current Science paper (Sriramachari, 2004).

The toxicological studies of blood also indicated the changes in blood pH, resulting in alterations in O_22 binding properties of Hb (Sriramachari et al., 1991). The exchange efficiency due to local clogging of the respiratory passages and alveolar surfaces also dampened the gas exchanges, due to functional alterations in Hb. Similarly, the lactic acid generated due to HCN inhalation, could have contributed to **'Metabolic Acidosis'**, which in turn affected delivery of O_2 to the tissues. This was further enhanced by freshly generated Pulmonary Edema found in the early autopsies of December 1984 as shown by marked increase in weight of the lungs and the brain. Even partial Carbamoylation of Hb would have affected removal of CO_2 and delivery of O_2 to tissues. It was presumed by some Clinicians, that after the end of 120 days of the life span of RBC, the physiological mechanisms would have been restored to normal in most of the cases studied.

The phenomenon of N-Carbamoylation also seems to have come to an end by the first Quarter (December 1984 - March, 1985). Likewise, as already pointed out, none of the other end-terminal amino acids of autopsy tissue proteins, were found to be positive after the first Quarter. Although thereafter, a few autopsy cases were found to be positive for N-Carbamoylation of Hb, even they could be rare instances of recycling.

Not withstanding Reports of Dr. Aggarwal in Dr. CR Krishnamurthy Report in 1987, the remote possibility of mutagenesis being perpetuated cannot be ruled out. In 1990, Knight had suggested that nucleic acid damage might produce mutagenesis and perhaps carcinogenicity. There is an obvious need for such long-term mutational and genomic studies in Bhopal.

Prof. Heeresh Chandra himself and some of his colleagues became victims of exposure and inhalation of toxic gases, initially in the open air of Bhopal and in the Autopsy Room of MLI. Eventually, he developed peripheral fibrosis of the lung which was confirmed by CAT scanning carried out in Delhi by common friend & Radiologist Dr. Sharma and published later in 1991. The CAT-scan was repeated after 3 years and demonstrated worsening, although no further

evaluation was available. The image was correlated with the autopsy finding seen in the cases of December, 1984. The visceral surface of lungs and liver, showed pin-head sized hemorrhages, particularly in the basal regions. It was first thought that these findings were in response to hypoxia. But subsequently they wee attributed to inhaled particulate matter obliterating the periphery of the lung giving rise to marginal fibrosis. They may be due to high molecular weight compounds like spiral compounds of molecular weight 269 (Chandra et al., 1994). This injury might have reduced the vital capacity of the lung.

In the Toxicology Project, attempts to characterize practically all the Chemical Constituents of the Tank Residue Analytes, with highly sophisticated GC-MS technique were more than successful. Although all the individual chemical constituents have not been traced in the autopsy tissues, the GC-MS findings suggest that one or more of them may be associated with the formation of Nitriles and/or release of the Isocyanate Molecules, leading to N-Carbamoylation. However, since subsequent autopsies did not reveal such compounds, it is felt that the early autopsies of the 1st Quarter could certainly be claimed as Forensic Evidence supporting the presence of inhaled tank residue constituents in the dead bodies of Bhopal Victims.

Before Bucher's publication in 1987, the only information regarding biological properties of MIC on health hazards in Human & Animals were by Kimmerle and Eben, (1964). The paper indicates that MIC by inhalation is poisonous to human beings, as defined by ICC regulations [a US company that offers advice on hazardous materials]. However, "No Apparent Injury" was reported by Union Carbide in 86 cases of MIC exposure, over a 9 year period (Sexton, 1973). But in the very subsequent year it has been stated that in spite of prompt treatment, major residual injury is likely (UCC Manual, 1974).

MIC is a strong poison, even on swallowing; the injury that is caused to the mucosa is because of its exothermic reaction when coming in contact with moisture of the tissues. The chemical and thermal necrosis seen in the lungs could be labeled as 'Burnt Lungs'. No bleeding from the nose was indicative of chemical coagulability of the blood in the lungs. The heat generated on inhalation also explains generalized Intra-Vascular Coagulation found inside the body.

Possible Mechanisms of MIC Toxicity: The foregoing account provides the physiological background underlying the reaction to injury of the victims, dead as well as living, exposed to the toxic Gases of BGD. The pyrolytic derivatives of MIC, viz., CO & HCN, etc. which inhibit the **'cytochrome oxidase c'** system of ATP cycle & also alter the function of Hb through formation of Met-Hb and CO-Hb. These two pyrolytic derivatives account for the phase of '***acute cyanide toxicity***' and Acute Respiratory Distress Syndrome (ARDS) and possibly extreme muscular weakness encountered in Bhopal. Naturally, the extent of pyrolysis of MIC and the quantum of each one of the above derivatives would largely depend on physical parameters like 'Hydration, Temp & Pressure' attained within the container and the external environment. Both these factors explain the phenomenon of rapid deaths encountered in the first 48 hours in BGD, which is also confirmed by elevated cyanide levels in cryo-preserved blood samples.

In addition, possible formation of the adduct of HCN~MIC (either through major or minor routes) results in delayed liberation of HCN or ***'sub-acute cyanide toxicity'*** with dramatic response to intravenous administration of NaTS, followed by restoration of muscular activity.

Recurrent Cyanide Toxicity: Variable degree or partial pyrolysis also explains a unique and new phenomenon of 'dual toxicity' reported in the Bhopal scenario. Apart from cyanide toxicity, direct toxicity due to MIC was confirmed by demonstration of N-Carbamoylation of 'End-Terminal Amino Acids' of blood & tissue proteins, yet another unique feature of BGD. Occasional ***'Recurrent Cyanide Toxicity'*** is probably unrelated to HCN, but due to interference of cyanide metabolism by the reversible and far more dynamic phenomenon of S-Carbamoylation following MIC crossing the 'alveolar capillary barrier', adequately established by Toxicology Group of Bhopal, contrary to the UCC's campaign echoed by a few scientist across the world. The phenomenon of MIC binding to end-terminal Amino Acids of Hb and a wide array of tissue proteins were established by the toxicological studies of Bhopal. Although, S-Carbamoylation could not be demonstrated by itself, because of lack of proper equipment, it is gratifying that Baillie and Slatter (1991) provided requisite crucial evidence.

Presence of other volatile compounds in the Exposees: Initially, Chemical & Instrumental Analysis could not reveal the presence of lighter compounds (like HCN, CO, CO_2, methyl amine etc) in the body tissues, although their presence was hypothesized as cause of the Cherry Red Discoloration of Autopsy Blood Samples. It was assumed that this was due

to presence of cyanogenic material in the body system. The presence of cyanide in the air was reported by Dr Max Daunderer (1984) and Central Air and Pollution Control Board (1985). Unfortunately, no method successfully described the presence of these compounds. A good reason could be due to technical difficulties and depending upon the sensitivity of the instruments available in those days. Later on, studies were carried out at MLI Bhopal, by developing new methodology on GC using "Head Space" techniques for the determination of Blood Cyanide Levels in the of exposed victims (Rao et al., 1989). Apart from the determination of cyanide, none of the other lighter molecules could be estimated.

MIC studies on Animals at IVRI: The presence of MA (Methyl Amine) and DMU (Di-Methyl Urea) residues were observed in the Meat & Milk samples of exposed animals by IVRI, Izatnagar. They also reported presence of CO in the form of Carboxy-Haemoglobin in the blood samples of exposed animals (IVRI Report, 1984-86).

Summary: An attempt has been made to discuss the physical nature of gas released, its impact on the environment and the physiological effects. The contribution of MIC itself and its thermal decomposition products (CO & HCN) leading to acute and delayed cyanide toxicity and MIC itself through N- & S- Carbamoylation leading to recurrent cyanide toxicity. The possible interplay and mechanisms of each category has been discussed. For proper understanding, the contribution of CO & HCN in BGD has been discussed from historical perspective of thermal decomposition of MIC (Blake & Ijadi-Maghsoodi) and current studies on 'Weapons Emissions' by artillery chemicals (Halperin., 2008).

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CHAPTER

6 STUDIES ON URINARY THIOCYANTE & CYANIDE

I. Urinary Thiocyanate

Early in the wake of the disaster of Methyl Isocyanate Gas Leak in Bhopal, there was a strong suspicion about the possibilities of death being caused by Hydrogen Cyanide (HCN). This was primarily based on the autopsy studies with characteristic Cherry Red colour of the blood and internal viscera like the lung and the brain, which was a prominent feature from the very beginning. These impressions of Prof. Heeresh Chandra were further reinforced by some preliminary information generated by Dr. Max Daunderer, a German Clinical Toxicologist who had visited Bhopal and carried out some laboratory tests on the samples of blood of survivors. It is also believed that he brought along with him to Bhopal over 15–20 thousands doses of $Na_2S_2O_3$ injections. It is not clear whether he did so either as part of Toxicologist's 'non-specific therapeutic repertoire' or specific information about cyanide-like toxicity of MIC and its potential response $Na_2S_2O_3$, available with him as a German Toxicologist.

Dr. Bisariya of Central Forensic Laboratory, CBI whose services were requisitioned, also examined many tissue and blood samples and indicated the presence of cyanide radical. The clinical evidence in support of venous blood being arterialized was reported by Dureja and Saxena (1987).This team of anesthetists was sent by Government of India and on 5th and 6th December 1984, who drew samples of blood from hospitalized cases.

Early Clinical Trials: Soon the use of Sodium Thiosulphate (NaTS) injections as an antidote was not only postulated by the visiting German toxicologist, Dr Max Daunderer, but strongly advocated by Prof Heeresh Chandra. In fact, even the Union Carbide in its earlier message suggested that in case Cyanide Poisoning was suspected, NaTS injections could be given in the standard manner i.e. along with Sodium Nitrite. However, for unknown reasons, very soon this message was withdrawn through the official channels (Mr. Dasgupta and Dr. Nagoo), even though NaTS was not a harmful treatment (Martindale, 1987. Dr BBL Mathur, Dean GMC, clearly mentioned in his report that Dr. Bhandari observed the relief on some of his patients with NaTS. Initial 5 cases admitted to DIG Bungalow hospital were treated with NaTS, when people were hospitalized and were disabled to get up from bed. Late Dr PS Narayanan gave 10cc of 10% NaTS by i.v. route. By the next morning all the patients could not only move about, but could do a few routine exercises they were asked to do. Dr Ishwar Das, then Health Secretary, Govt. of MP, was a witness to this miraculous therapy. Even then, at government level, he did not support the treatment. Subsequently, therapeutic role of NaTS was established by the epidemiological studies of ICMR (ICMR Report, 1986). Even the post-mortems, after a lapse of 10-12 days, continued to show similar gross features of Cherry Red Discoloration. It was at that stage that the team from the Institute of Pathology led by Dr S Sriramachari, Dr HMK Saxena and Dr B Dasgupta were associated with the post-mortem studies and also with the toxicological investigations. which were being pursued vigorously in the Medico Legal Institute by Prof Heeresh Chandra.

Preliminary Laboratory Trials: The several possible causes for the reddish discoloration of the blood and viscera in the context of the Bhopal Gas Disaster were critically examined. Direct Vision Spectroscopy of Blood did not reveal any evidence of Carboxy-Haemoglobin, nor was there any evidence suggestive of Meth-Hb in autopsy samples of blood. Instead, standard bands suggestive of Oxy-Hb were observed. Our experiment with Drager tube inserted into the slit trachea, gave positive result for presence of HCN at a level of 2.25 ppm. These findings lent further support to the possible role of Hydrogen Cyanide being responsible for the gross reddish discoloration of the organs. Direct Vision Spectroscopy of autopsy samples of blood revealed only standard bands suggestive of Oxy-Hb, but there was no evidence Carboxy-Haemoglobin nor Meth-Haemoglobin.

However, as briefly stated in the previous Section on Patho-Physiology, Kamath and Associates in their exhaustive Clinical Studies of survivors in and around the Bhopal Railway Station reported the continued presence of Meth-

Hb. Instead of ignoring the variability of this phenomenon, it might be possible to interpret it as part of the localisation of the spectrum of changes due to variations in the dispersal of the gaseous cloud spread over Bhopal. The relatively lighter CO of the Gaseous Cloud or Plume might have been differentially distributed over Bhopal City, which accounted for of a minor but unique Patho-Physiological imprint of the regional or terrestrial concentrations of a spreading mixture of lethal Toxic Gases across a City.

Genesis of Urinary Thiocyanate Studies: As early as 18th of December, 1984, while an autopsy was being carried out, Dr. Sriramachari suggested that since sufficient time had elapsed for the HCN to be detoxified or neutralized by the natural enzymatic systems such as Rhodanese of the liver, it might be worthwhile examining the samples of urine of the dead bodies for the presence of elevated levels of SCN^- (urinary thiocyanate). Thus, right from the beginning, the possible role of slow reactions within the body resulting in accumulation of cyanide-like radicals was postulated. It was neither swayed by determined opposition to the use of Sodium Thio-sulphate on the one hand nor did it acquiesce to populist demands for universal and large detoxification of the entire population. Unlike the classical 'cyanide pool hypothesis' advocated in RT William's Book on Detoxification Mechanisms (1959), an alternate working hypothesis of a more likely "Cyanogen Pool" was clearly enunciated in December 1984 itself.

Standardization of Urinary SCN^- Estimations: Recalling some noteworthy earlier studies way back in 1954, on Extra-Cellular Space etc., at NRL, Coonoor, the fore-runner of NIN, Hyderabad, Sriramachari obtained "by wireless", full details about Bowler's method for estimation of thiocynate concentrations in biological fluids. Since INMAS, in New Delhi, was also known to carry out SCN^- estimations in serum, though not in urine, the help of Brig. Lakshmipathy and colleagues, was sought for a preliminary comparative study on Normal Controls along with suspected samples of Bhopal. It was found that the values ranged from 0-0.5 mg%, where as in 14 out of 54 Bhopal samples, the levels were above 0.5 mg%. In 5 out of these 14 samples, the values were more than 1 mg% and the rest between 0.5-1 mg percent. Hence as an immediate measure, it was decided to take a value of double the normal maximum, as a cut-off point, for monitoring urinary thiocyanate studies in the Bhopal Gas Victims.

Simultaneously, steps were taken to generate data on normal urinary levels in unaffected areas of Bhopal and nearby places like Hoshangabad, Sehore, etc (Table 6.1).

Table 6.1. Periodical study of Control Urine Thiocyanate

Period	No. of Cases	mg % *		mgs (24 hours) **	
		non-smokers	smokers	non-smokers	smokers
Month & Year	**N**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
March 1985	410	0.528 ± 0.190	0.846 ± 0.178	6.152 ± 2.094	8.676 ± 1.505
April 1985	420	0.523 ± 0.193	0.879 ± 0.225	6.152 ± 2.094	9.066 ± 1.888
October 1985	63	0.508 ± 0.187	0.755 ± 0.279	6.172 ± 2.183	8.803 ± 2.812
November 1985	31	0.409 ± 0.158	0.675 ± 0.305	4.923 ± 2.497	7.265 ± 2.622
June 1986	50	0.549 ± 0.228	0.716 ± 0.211	4.494 ± 2.630	4.398 ± 1.581
March 1987	20	0.636 ± 0.170	-	6.511 ± 1.091	-
May 1987	85	0.582 ± 0.206	0.860 ± 0.209	6.209 ± 0.209	9.254 ± 1.672
Total	**1079**	**0.531 ± 0.195**	**0.836 ± 0.216**	**6.048 ± 0.216**	**8.651 ± 1.991**
't' Value		*** 20.848**		**** 16.958**	

From December 1984, this method provided by Dr Sriramachari was standardized by Dr Ramaiah for routine analysis employing Bausch & Lomb Model UV-Vis-710 Spectrophotometer. The test reaction mixture contained 2ml of the urine, 0.5 ml of 20% TCA (trichloro-acetic acid), which were mixed thoroughly and allowed to stand for about 10 min for completion of the reaction. Addition of 2.5 ml of 16% ferric nitrate in nitric acid reagent gives the characteristic colour and was studied spectrophotometrically at 470 nm, against the reagent blank of the same sample. Calibration curve was plotted by measuring OD of different concentrations of standard solution of sodium thiocyanate.

Cross Checking of SCN^- Estimations: In view of the general controversy about Cyanide Toxicity, every care was taken to counter-check the urinary SCN^- values obtained by Bowler's (1944) method with several newer methods, like

HSGC (Head Space Gas Chromatographic) method of Feldstein and Klendshoj of (1954), as recently modified by Jallageas in 1984. Prof. Heeresh Chandra *et al* confirmed to the hilt the results by Bowler's method which was later on published (Rao et al., 1989). This method was used to read abnormal samples which were not measurable by Bowler's spectrophotometric method. In between the analysis, many methods were developed on micro-diffusion and on GC, HSGC for the reliability of the conventional Bowler's Method. Although this method is old, it is simple and gives predictable and reproducible results; therefore, a suitable number of control samples were also studied. Analysis clearly distinguished between smoker and non-smoker groups and persons who consumed 'cyanogenic vegetables' and some of the interfering medicines (Rao et al., 1989).Thus the purpose of assisting treatment or management of the Bhopal Gas Disaster victims was largely accomplished. A summary of salient findings are presented in the accompanying Tables of this section.

Extended Toxicological Studies: This work was successfully initiated both for determining the pre- and post-injection levels with a view to assess the response to NaTS injections. A large number of patients showed 3-4 fold increase in the urinary thiocyanate levels, as compared with the upper limit of 0.5 mg%. In those early days the response to NaTS was so dramatic that patients who were in a helpless situation were suddenly relieved of the major symptoms.

Double Blind Clinical Trial (DBCT) studies: Towards the end of January, 1985, in view of the violent and diametrically opposite views on the use of NaTS injections, the ICMR undertook the first Double Blind Clinical Trial (DBCT) study (***Annexure 6.1***). It was followed by monitoring of cases given in the course of injections. There was clear-cut statistically significant evidence that concomitant with clinical improvement, there was marked elevation of urinary SCN^- levels, following the administration of NaTS injections. This evidence constituted the bedrock for its use and also as a guideline for its subsequent use. The ICMR through its Toxicological Teams pursued the problem vigorously for the next 4 years.

It may be pointed out that during the years 1985-88 several organised studies had been undertaken apart from over 18,000 patients whose urine had been tested before, during or after the treatment with NaTS injections as shown in the accompanying Table 6.2.

Table 6.2. Urinary Thiocyanate Values of Exposed Population up to October 1987

	No. of Cases	Mean ± SD (mg%)
24 hours	6834	0.9897 ± 0.5615
12 hours	774	0.8966 ± 0.4601
Spot	2859	1.1382 ± 0.7903
Deep Freeze (Autopsy)	190	1.0720 ± 0.5765
Claim	3059/3524	0.7807 ± 0.5054
Pre-injection	1004/1253	1.0744 ± 0.7652
Post-injection	832/1253	1.2644 ± 0.9781
Could not be detected	1975	-
Total	**17527**	
Control	**1079**	**0.598 ± 0.205**
Grand Total	**18606**	

The noteworthy findings are as follows:-

a. A series of DBCT studies were carried out on patients administered either one or more injections of NaTS or a harmless control substance like glucose.

b. There were a large number of Hospital-based Studies undertaken both in the Hamidia Hospital and in the 30-bedded Hospital, since known as the Jawaharlal Nehru Hospital in a project under Late Dr PS Narayanan. In the latter, a large number of patients, both children as well as adults, had been followed up over a long period of time along with their response to treatment with NaTS as judged by urinary SCN^- levels.

c. In addition, a large number of Community Based Investigations on the Gas Affected Population have been studied and analysed on a quarterly basis during the years 1985-86 and 1987. Several hundreds of Controls from the 'unaffected areas of Bhopal' and from different batches of Army Jawans located in Bhopal, were also investigated for

their urinary thiocyanate levels. In all these studies several factors have been meticulously examined taking into account smoking habits, consumption of vegetables rich in cyanogenic materials, etc (Table 6.1).

d. The bulk of these studies have revealed that indeed there was an elevation in the urinary SCN^- levels in the gas affected population and that there was a prompt therapeutic response to NaTS. However, by the end of 1985 and the first Quarter of 1986, the values had tended to become 'Normal, with Poor or No Response' (Table 6.3 a, b & c).

Table 6.3a. Paired 't' Test on Age and Sex Based Groups

Group	Cases(n)	Values of Urinary Thiocyanate in		't'	Significance
		1985-86	1987-88		
A. Below 40 years Male	44	1.2354±0.4554	0.8931±0.4054	4.5197	99.9%
B. Below 40 yearsFemale	41	1.2178±0.5446	0.7066 ±0.3817	5.4961	99.9%
C. Above 40 years Male	36	1.0341 ±0.7574	0.8229 ±0.3755	3.4645	99.9%
D. Above 40 years Female	12	1.0341 ±0.4508	0.7166 ±0.2525	2.0631	Insignificant

Group	Cases(n)	Values of Urinary Thiocyanate in		't'	Significant
		1987-88	1988-89		
A. Below 40 years Male	44	0.8931±0.4054	0.8833 ±0.3948	0.213	Insignificant
B. Below 40 yearsFemale	41	0.7066 ±0.3817	0.7768 ±0.3090	0.9141	Insignificant
C. Above 40 years Male	36	0.8229 ±0.3755	0.7431 ±0.3567	0.9223	Insignificant

Table 6.3b. Paired 't' Test on Groups Based on Smoking Habits

Group	Cases(n)	Values of Urinary Thiocyanate in		't'	Significance
		1985-86	1987-88		
E. Smokers	22	1.2154 ±0.4967	0.7740 ±0.4967	3.8980	99.9% Significant
F. Tobacco chewers	28	1.1889 ±0.4318	0.8771 ±0.3815	3.8771	99.9% Significant
G. Non-smoker Non- Tobacco chewers	47	1.2546 ±0.5547	0.7770 ±0.3470	4.9506	99.9% Significant

Group	Cases(n)	Values of Urinary Thiocyanate in		't'	Significant
		1987-88	1988-89		
E. Smokers	22	0.7740 ±0.4967	0.7773±0.3092	0.264	Insignificant
F. Tobacco chewers	28	0.8771 ±0.3815	0.8979 ±0.3611	0.926	Insignificant
G. Non-smoker Non-Tobacco chewers	47	0.7770 ±0.3470	0.7364 ±0.2877	0.6189	Insignificant

Table 6.3c. Paired 't' Test of Groups Based on Urinary Thiocyanate Values

Group	Cases(n)	Values of Urinary Thiocyanate in		't'	Significance
		1985-86	1987-88		
H. Previous values of more than 1 mg%	88	1.4538 ±0.3760	0.8364 ±0.4040	11.2143	Highly significant
I. Previous values of less than 1 mg%	45	0.6627 ±0.2174	0.7134 ±0.3549	0.1929	Insignificant

Group	Cases(n)	Values of Urinary Thiocyanate in		't'	Significant
		1987-88	1988-89		
H. Previous values of more than 1 mg%	88	0.8364 ±0.4040	0.7984 ±0.3515	0.655	Insignificant
I. Previous values of less than 1 mg%	45	0.7134 ±0.3549	0.6944 ±0.2935	0.277	Insignificant

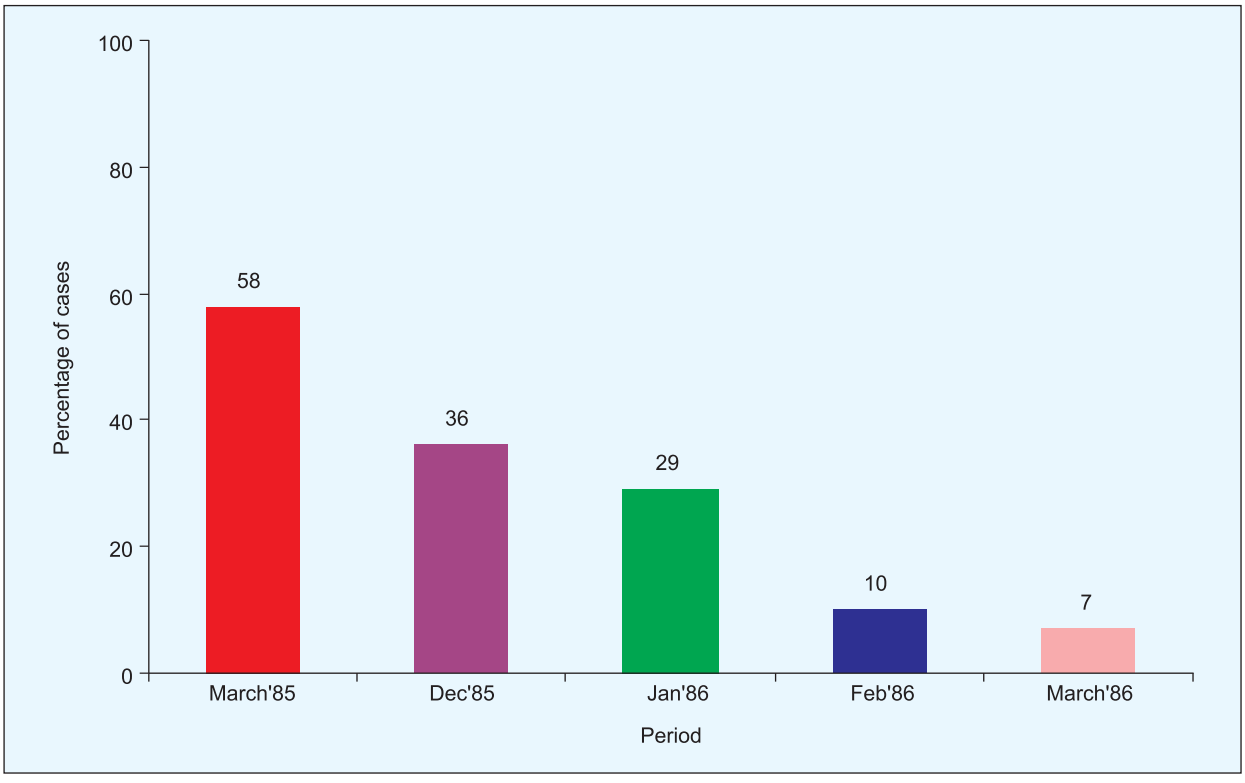

Figure 6.1: Increase in the percentage of cases above 1 mg% two hours after NaTS injection.

This is borne out by the sequential information that had been collected over representative samples as already stated. Hence it was felt that in view of the declining trends, the need for NaTS injections should be progressively tapered off (Figure 6.1). Based on such evidence, a majority of the members of a committee appointed for the purpose by the Supreme Court recommended that there was no further need to administer NaTS for detoxification.

From time to time seven control urinary thiocyanate studies were made in MLI under the Project and a total of 1079 cases were studied. They were put to a strict drill of 24 hour collection of urine i.e., from 10 am to next day 10 am and were fed with or without the thiocyanate rich diet viz., cauliflower, cabbage or vegetables of cruciferous family etc. These studies revealed that the urinary thiocyanate excretion is 0.531 mg% (SD = 0.195) with an average of 6.048 mg% per day (SD = 0.216) with an average of 8.651 mg% per day (SD = 1.991) among 839 non-smokers and 240 smokers.

Urinary thiocyanate levels in exposed population were found higher than controls which showed a further rise after giving NaTS injection giving relief to the patients. After first injection of NaTS, percentage of cases above 1 mg% increased by 58.0% in the early 1985 which gradually came down to 7.0% in 1986. A total of 17,527 urine samples have been analysed till October 1987 followed by 1350 samples till June 1988 (Table 6.2). Further Details are available in the document which had been submitted to Supreme Court (***Annexure 6.2***).

II. Cyanide Studies

It is possible that hydrogen cyanide, which is slightly lighter than air could have descended down in the Aerosol and was consumed by the victims with acute fatal results. Part of the HCN, especially in a cold trap of the prevailing temperature, might have been bound to the twice-heavy Methyl IsoCyanate, a distinct possibility in the light of the observations of Blake and Ijadi-Maghsoodi, 1982 in their '***landmark paper'*** on Kinetics and Thermal Decomposition of Methyl IsoCyanate. It is beyond doubt that HCN would have been released as a breakdown product, when Methyl IsoCyanate got heated, more so under pressure (UCC, 1985; Blake and Ijadi-Maghsoodi, 1982; Bhattacharya et al (DRDE, 1988).

Analysis of aqueous extract of tank residue material at ambient temperature for the 'free cyanide' was studied by HCN Detector Tubes, as well as by Spectrophotometry of chromometric reagents (APHA, 1960; Pettigrew et al., 1973; Jallageas et al., 1984; Upadhyaya et al., 1984). Even five years after the BGD, a clear positive indication was observed by

detection tubes of HCN and also on spectrophotometry. Paramethine gluconic aldehyde gave absorption maxima at 400nm with anthranilic acid, at 530nm with benzidine, 590nm with barbituric acid and 490nm with p-Phenylene-Diamine. Tank residue showed presence of free cyanide at 0.05 percent. On GC-FID, using Super-Q column, a clear Cyanide Peak was detected. Thus, by both Spectrophotometry and Chromatography, presence of Cyanide was detected in the Tank Residue. Since it is lighter than air, some part of it might have escaped from the Tank Residue and its vicinity. Only that fraction, which was adsorbed or entrapped within the tank residue material, was detected. Possibly at the time of the accident, along with other volatile compounds, the concentration of gaseous HCN would have been much higher.

The raised thiocyanate levels in the urine noticed in the victims and gradual tapering off with the passage of time, as against Controls, is further proof of disturbance of cyanide metabolism in their bodies. The Epidemiological Studies conducted by ICMR in 1986 which surfaced in 1988, that clearly brought out the therapeutic response to NaTS therapy, suggested an enlarged "Cyanogen Pool" in the body. This hypothesis was further strengthened when the studies were repeated after a provocative dose of NaTS, accompanied by raised urinary thiocyanate in the 'Exposed Population', as against Controls. NaTS therapy brought excellent response in the victims (BGDRC 1987). But subsequent re-elevation of urinary thiocyanate values, with-reappearance of the symptoms, although of less intensity, continued to be an enigma.

Sodium Thio-Sulphate is not an ideal antidote of acute cyanide poisoning as it does not run parallel to the tissue distribution of HCN due to its limited membrane permeability (Way, 1984). Cyanide gets converted to thiocyanate in the presence of enzyme 'rhodanese' (***thiosulphate sulphur transferase***). But the conversion of thiocyanate to cyanide has also been reported in the literature, not due to 'transferase activity' by rhodanese, but because of an enzyme called "thiocyanate oxidase" (Goldstein & Rieders, 1953; Cohen & Oppenheimer (1971). This enzyme is found in erythrocytes in a very good amount, while only traces of rhodanese are found in those cells. Thiocyanate oxidase appears to possess an optimum pH 7.4 and is not inhibited by alkaline earth ions as is Rhodanese. This thiocyanate oxidase converts thiocyanate to cyanide. Perhaps one or more of these factors are responsible for the recurrence of Chronic Cyanide Toxicity and the response to NaTS eventually tapers off. Secondly, Rhodanese is specific only for 'free cyanide' and has no action upon organically bound 'cyano-groups', such as those in acetonitrile, propio-nitrile, cyano-acetic acid, cyanamide, etc. Although, these compounds may first need to be converted to HCN by metabolic activities, they are later excreted in the form of thiocyanate (Williams, 1959). There are variety of other substances which react with cyanide, besides Thiosulphate; however these reactions are usually reversible (Sorbo, 1975; Westly, 1980).

The extreme toxicity of cyanide is thought to be due to the ability of the ion to tie up active coordination positions of metal atoms in one or more enzymes present in very small amount in the body but are indispensable for metabolism. A number of cyanide complexes are so slowly dissociated that they show the reactions neither of the metal ion nor of the cyanide ion. The most stable cyanide complexes are being formed by those "metals lying between chromium and zinc" in the Periodic Table like Mn, Fe, Co, Ni and Cu. Since Fe is present in Hb, Cu is important for the synthesis of Hb and Mn, Ni & Cu are enzyme activators; all these activities may be suppressed as cyanide binds all these metals to form a stable complex.

A number of valency states of metals, thought too unstable to exist in the solution as ordinary ion, are greatly stabilized by the strong coordinating action of cyanide ion. Such cyanide complexes of Cu(I) are known, although simple salts of Cu in this oxidation state do not survive in the solution. The reaction of cyanide ion with divalent Cu(II) may be mentioned here, where cyanide acts in a dual capacity, both as a complexing agent and as a reducing agent. If sufficient cyanide is present, part of the cyanide will be oxidized to **"CYANOGEN"** and the remainder will attach itself to the Cu ions formed in the solution:-

$$2Cu^{++} + 8CN^{-} \rightarrow C_2N_2 + 2Cu(CN)_3^{--}$$

One metello-enzyme cytochrome oxidase is very sensitive to cyanide, as it contains Fe^{2+} which binds with cyanide and blocks the tissue utilization of oxygen, thus producing histotoxic anoxia (Way, 1984). However, it may be pointed out that there are some other enzymes also which are either equally or more sensitive to cyanide as cytochrome oxidase (Solomonson, 1982). Cyanide is reported to react with 42 enzymes in vivo (Patty, 1963). Therefore, cyanide toxicity may not be a single 'Biochemical Lesion', but a complex effect on various enzyme systems involving Schiff base intermediates as well as metallo-enzymes. Studies using radioactive glucose indicated that cyanide alters glucose catabolism, resulting in a 100 percent increase in conversion of glucose by pentose phosphate pathway. This shift results in a

decrease in ATP/ADP ratio (Albaum et al., 1946) and an increase in NADPH. The increase in NADPH represents a balanced redox state to compensate an enhanced conversion of pyruvate to lactate at the expense of NADH (Jacob and Diem, 1974).

The recurrent sign of cyanide poisoning suggested that the acrylonitrile or other 'Nitriles' (possible compound formed and present in the Tank Residue) or one of their metabolites, are stored in the tissues and from those storage spaces, cyanide may be slowly released and may cause recurrent problems. It emphasizes that the prolonged treatment with antidotes for a peculiar type of chronic cyanide poisoning may be required and that many doses of such antidotes can be given safely over a prolonged period. The toxicity of acrylonitrile may solely be attributed to the liberation of cyanide (Vogel, 1984). Magos (1962) suggested that full acrylonitrile molecule might have direct toxicity.

Likewise the presence of several known and unidentified derivatives of MIC detected in the Tank Residue could also play a contributory role in recurrent cyanide toxicity. It is also possible that that interaction of chloroform and MIC may give rise to Chloro- derivatives of MIC suggested by Romonenko (2001) might be responsible for long term toxicity. So, Sriramachari and Jain, along with Dr. Vijayaraghavan and Dr. Bhattacharya, have currently initiated a Research Project on Chlorotropism of MIC at DRDE, Gwalior.

The chronic poisoning from cyanide exposure has also been discussed by other workers; Wolfsie and Shaffer, 1959). Sandberg (1976) pointed out that the chronic cyanide poisoning indicates delayed-neuropsychiatric problems. During detoxification mechanism cyanide is converted to thiocyanate, which is also a toxic compound, beyond certain limits. Organic thiocyanates are more dangerous than inorganic thiocyanates and are probably converted *in vivo* to cyanide, causing the recurrence of symptoms (Kaye, 1977).

An independent but parallel study carried out by late Dr. PS Narayanan and others under ICMR project on "Studies on Pulmonary Function Tests and Blood Gas Analysis" in 1986-87, brought out parallel results. It indicates definite downward trend in the urine thiocyanate excretion (Tables 6.3a, b & c). This collaborative study strongly indicated possible body storage of cyanogenic material being released over several months causing excretion of high urine thiocyanate and symptoms of weakness. Their study further concluded that the patients returned to normal levels over a period of three years since the exposure. It was concluded that the cyanogenic material was gradually depleted from the body (BGDRC, 1989).

Blood and Tissue Cyanide: Cyanide was determined in the blood qualitatively by a colour test using micro-diffusion technique followed by spectrophotometer (Jallageas et al., 1984). Attempts were also made to detect cyanide by a head space gas chromatographic method as described by McAuley and Reive (1983).

In an autopsy Prof Heeresh Chandra demonstrated presence of HCN trapped in the lungs. In this method nose and mouth were packed with wet cotton. A small oblique incision was made in the neck along the trachea. The Drager tube was inserted in the trachea and chest compressed as is in artificial respiration. The Drager tube indicated HCN, by change of colour.

Spectrophotometric method using Micro-diffusion Technique: One ml of blood or 1 g of macerated tissue was taken in the outer chamber and 2ml of 0.1N NaOH in the inner chamber. The unit was sealed with paraffin wax in case of glass microdiffusion dishes and sealed with liberating agent in case of three chambered polymer dishes. 10% H_2SO_4 was used as a liberating agent and 3-4 hr time was given for complete diffusion. One ml of cyanide trapped NaOH was taken to a stoppered test tube for the test while 1 ml of 0.1N NaOH was used as a blank. To it 2 ml of 0.6N HCl was added followed by 0.5 ml of saturated bromine water. Five minutes were given for the complete bromination. Excess bromine was neutralised by 2% sodium arsenate in 0.1N NaOH and 3 ml reaction mixture consisting of benzidine and pyridine and colour was read on spectrophotometer at 530 nm. A standard graph was plotted from the standard cyanide solution (Jallageas et al., 1984).

Gas Chromatographic Method: Four ml blood or 4 g tissue was taken in a vial and 2 ml of acetic acid was added and sealed with Teflon tape. Hydrogen cyanide generated was subjected to GC using head space technique. Retention time was checked with that of standard solutions of KCN. GC conditions were as follows - Gas chromatograph Perkin Elmer model Sigma 300, column Porapak-Q 80/100 mesh 12' x 1/4" OD glass column, nitrogen 25 ml/min, FID 230°C, Inj 210°C and Oven 125°C. Retention time was noted to be 7.2 min (Mcauley and Reive, 1983).

To overcome limitations of this method, a new method was developed by Rao et al., in 1986, modifying the methods

described by Funazo et al. (1981). This method was found to have 5.0µg% lower limit of detection. The Coefficient of Variation decreased from 9.7 µg% at 10 µg% to 3.2% at 800µg%. For routine analysis Jallageas method (1984) was used. Fifteen control samples were found to be in the range of 5 to 30µg% with an average of 20% while in control post mortem blood of 31 cases it was a little higher accounting 25µg% average in the range of 10 to 15 µg%. Thirty four blood samples from exposed OPD patients were found to be elevated following the range of 50 to 110 µg% with an average of 70 µg%. More elevated levels were recorded in post mortem gas exposed cases of 43 blood samples with an average of 150 µg% in the range of 60 to 360 µg% (Table 6.4).

Table 6.4: Blood Cyanide Levels of Control and Gas Exposed cases of Bhopal

Group	No. of Cases	Cyanide Range (µg/dl)	Medial value (µg/dl)	S.D.	Coefficient of Variation (CV)
Control Blood (Living)	15	5-30	20	7.825	46.643
Control Blood (Postmortem)	31	10-50	25	10.988	40.555
Gas Exposed Blood (Living)	34	50-110	70	23.233	34.850
Exposed Blood (Postmortem)	43	60-360	150	73.243	61.257

- Some of the samples i.e.21 of S. No. 3 and 16 of S. No. 4 were studied after preservation (up to two months and more) and found no remarkable difference in preserved samples cyanide level.
- All samples were preserved in Sodium fluoride at -200°C.

The Bhopal Scenario: Blood samples of 59 autopsies, 10 samples of OPD and 43 autoposy tissues like liver, lung, spleen, kidney, brain from cases having history of gas exposure have been analysed with appropriate control samples. Post mortem samples of 23 cases of blood and 12 tissues belonging to 5 post mortems gave positive results. Cyanide was quantified to be equal or more than 250µg%, since this method is not sensitive below this concentration, therefore we switched over to the other sensitive method.

The Cherry Red blood was first observed in December 1984 post- mortems of aerosol affected persons (BGDRC, 1985). It was then hypothesized by Prof. Heeresh Chandra and colleagues that HCN could have been released; because it is well known that in cyanide poisoning the blood remains 'Cherry Red'. The underlying mechanism is due to the poisoning of tissue **cytochrome oxidase** and consequent inability to utilize O_2. Thus as a result of failure of tissue respiration, oxygenated Blood Hb fails to shed its oxygen load. As a result of hypostasis, the colour of the whole body is also pink. So also the eyes appear to be injected red. Initial **GC-FID** studies were carried at Medico Legal Institute, Bhopal, to look for the presence of Cyanide in the victims. Although Cyanide Metabolism exists in the human body (Arena, 1986), its function and utility are not exactly known. The normal values ranges from 15 µg to 30 µg per 100 ml of blood. The values were found to be of much higher range both in the autopsy samples as well as clinical cases indicating some degree of cyanide poisoning in the exposed victims at the different stages. The raised values to this single exposure were found in the samples until the end of 1985 (Table 6.4), as stated by BGDRC (1987). The above analysis was presented in the Brain Storming session organised by Dr. Krishnamurthy Commission in 1987.

Thus to conclude, from the very beginning it was felt necessary to determine the actual cyanide content of blood and tissues of dead and living victims exposed to BGD. Indeed the efforts have more than fulfilled the expectations. In the face of such incontrovertible evidence generated, why and how such a tirade was mounted and sustained against Cyanide Toxicity & NaTS Therapy of BGD by variegated/different groups of Indian Clinicians, Administrators and even Activists in India and abroad, remains an inexplicable enigma even as of today! It is one of the reasons for placing on record the entire evidence for future re-appraisal of a rare scientific phenomenon following a unique Chemical Disaster.

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❑

First Double Blind Clinical Trial (DBCT) on the Excretion of Thiocyanate by Patients Exposed to the Bhopal Gas Tragedy in Response to Intravenous Injection of Thiosulphate

By **Indian Council of Medical Research**

A great industrial disaster unprecedented in history took place at Bhopal on December, 1984 resulting in the immediate deaths of at least 2500 peopl;e and large number of cattle, sheep and other animals. It resulted from the leakage of methyl isocyanate at high pressure and perhaps at high temperature from a storage tank of union carbide factory. The immediate treatment of these gas victims was uncertain. Dr. Max Daunderer who visited Bhopal in Dec. 1984 suggested intravenous injections of thiosulphate as treatment for these individuals based on the positive evidence of presence of cyanide in patients, he tested by Drager tube. This test is however not specific.

Dr. Sriramachari felt that one of the simple tests for cyanide poisoning is the estimation of thiocyanate in the serum or its elevated excretion in the urine following an injection of thiosulphate. In order to test this idea a double blind evaluation study of thiosulphate injection to patients of gas victims on the urinary excretion of thiocyanate was carried.

The results presented in this paper show that intravenous injection of thiosulphate to the gas victims resulted in elevated excretion of thiocyanate in the urine. The beneficial effect of thiosulphate injections to the gas victims may thus be associated to excretion of cyanide radical as relativity nontoxic thiocyanate radical.

Materials and Methods

All chemicals that were used in this study were of analytical reagent grade.

Estimation of Thiocyanate in Urine: The method used for the estimation of thiocyanate in the urine is adopted from the method used for estimation of thiocyanate in serum, as modified by Bowler. The procedure in brief is as follows.

A total reaction mixture of 5 ml. contained at final concentrations, trichloroacetic acid 2%, ferric chloride 8%, nitric acid O.5N and urine volume 0-2 ml. The orange color that developed in presence of standard solution of potasium thiocyanate or thiocyanate present in added urine was measured in spectrophotometer at 470 nm. The color intensity is linear upto 0.1 mg potasium thiocyanate in the test solution and sensitive enough to detect as low as 0.01 mg as potasium thiocyanate. In order to avoid any interference by the color of the urine sample on thiocyanate estimation by the method, the corresponding blank contained all as in test but ferric nitrate.

The contribution of color by ferric nitrate alone against water blank was found to be negligible. The level of thiocyanate in urine was expressed as mg% as potasium thiocyanate.

Design of Double Blind Study of Thiosulphate Injection to Gas Victims on the Excretion of Thiocyanate in their Urine.

Selection of Patients: Patients who were exposed to gas tragedy of 2nd & 3rd of Dec. 1984 and suffered prolonged problems and who had no cardiac disease or any infections were included in the study. Patients who were between ages 20 to 40 were taken without any sex discrimination.

In the first room, patients were examined and assessed and allotted a number. This room could not be seen by other investigators. Dr. Nanda and Dr. P.S. Narayanan performed the case-noting and allotted IAI number to the patients. The patient was sent to Dr. A. Jain in a different room who was allowed to take the blood samples, urine samples and send them under a new 'B' number to the third room to Dr. Ramaiah, only with the numbers. Dr. Ramaiah and Dr. Jain ", were not allowed to examine the patients. Dr. Jain administered placebos and drug on her own random selection, which was not brought to the knowledge of Dr. Narayanan. Dr. Ramaiah performed the various measurements.

In this way 30 patients were tested over a period of 3 days. At the end of these analyses the code was deciphered.

Results

The thiocyanate level in the urine was estimated 1 hr. after intravenous injection of either glucose or thiosulphate (10 ml of 10% solution). A total of 3 injections were given in a day. These results are presented in Table 1. It can be seen from column 3 of Table 1 that a 50% or more increase in the excretion of thiocyanate in the urine occurred in only 3 out of 13 patients who were given intravenous injections of glucose while 10 out of 14 patients who were given intravenous injections of thiosulphate showed elevated excretion of thiocyanate (Table 1). These differences are statistically significant. These results thus clearly indicate that intravenous injection of thiosulphate rather than glucose to the gas victims, results in the increased excretion of thiocyanate.

Effect of multiple intravenous injections of thiosulphate to gas victims every two hrs. on the excretion of thiocyanate in their urine.

The level of thiocyanate in the urine 1 hr. after intravenous injection of thiosulphate, 1 hr. after 2nd intravenous injection of thiosulphate and 1 hr. after 3rd intravenous injection of thiosulphate to a gas victim was estimated and the results are presented in Table 2. It can be seen that the peak excretion of thiocyanate by the gas victims occurred 1 hr. after either 1st, 2nd or 3rd intravenous injections of thiosulphate but more often 1 hr. after the 3rd intravenous injection of thiosulphate. The level of thiocyanate in the urine of gas victims 1 hr. after and 1 hr. after 2nd intravenous injection of glucose was determined and the data are presented in Table 3. It could be seen that only in one case out of 28 determinations the level was 2 mm while in the case of thiosulphate injection to victims under similar conditions as many as 9 determinations out of 30 showed thiocyanate levels in the urine 2 mg% or above indicating again that intravenous injection of thiosulphate to gas victims results in higher excretion of thiocyanate in their urine. Injection of Thiosulphate to normal individuals did not increase thiocyanate excretion in urine.

In order to see whether intravenous injection of thiosulphate to normal individuals unexposed to the gas tragedy but living in the same city and neighborhood results in elevated excretion of thiocyanate, five doctors who volunteered were given intravenous injection of thiosulphate and the thiocyanate level in the urine before and 1 hr. after injection was tested and the results are presented in Table 4. It could be seen that in no case a 50% increase in thiocyanate level after thiosulphate injection was observed while such increase occurred in 10/14 of gas victims (Table 1) suggesting that only in gas victims intravenous injection of thiosulphate resulted in the elevated excretion of thiocyanate. The levels of thiocyanate in the urine of the gas victims even without thiosulphate injections appear to be on the higher side than the normal values reported in literature. This is further supported by the estimation of thiocyanate in the urine of individuals located in different areas of Bhopal. These results are shown in Table 5. It can be seen that only 33% of people living in areas unexposed to the gas leakage had more than 1mg% of potasium thiocyanate equivalent in their urine compared to 65% of people living in area exposed to the gas tragedy.

Discussion

The data presented in Tables 1-5 clearly show that the population exposed to the gas leakage in Bhopal during Dec. 2nd, 1984 were excreting higher than normal levels of thiocyanate in their urine (Table 5) and that this excretion is further elevated by the intravenous injection of thiosulphate rather than glucose (Table 1). Thiosulphate had no such effect when given to individuals unexposed to the gas tragedy (Table 4).

Thiosulphate is well known antidote for cyanide poisoning and it is a sulfane donar in the conversion of cyanide anion (CN^-) to relatively non-toxic thiocyanate anion (SCN^-) by rhodanese present in liver, red blood cell and most tissues in man. Thiocyanate is excreted in the urine. Multiple injections of cyanate over many months to rats, rabbits and monkeys do not result in any increase in the cyanide (CN^-) level in the serum. However, no information is available on the blood level of cyanide in animals exposed to methyl isocyanate which is more reactive than cyanate and therefore much more toxic than cyanate.

In view of the fact that thiocyanate was estimated by a colorometric procedure, one may argue whether what is actually measured by the method described here is due to thiocyanate itself or some metabolite which was excreted in larger quantities following the intravenous injection of thiosulphate to gas victims. This appears unlikely since thiocyanate levels in the serum estimated by this method were fairly comparable to the levels obtained by more specific

method for the estimation of thiocyanate. In absence of uncertainty to what exactly the gas victims inhaled in addition to methyl isocyanate it is difficult to give a specific mechanism how thiosulphate increased the excretion of thiocyanate in the urine of gas victims. But it appears possible that they either inhaled cyanide along with methyl isocyanate or by as yet unknown mechanism methyl isocyanate inhaled by the patients could have resulted in the generation of cyanide ions and these are converted to thiocyanate ion by rhodanese in presence of thiosulphate.

Table 1. Change in level of thiocyanate in urine after an injection of glucose or thiosulphate only. Thiocyanate level is expressed as potasium thiocyanate in mg%

1 hr after glucose injection (1)	1 hr after 2 injection of glucose (2)	Percent change from preceding value (3)	1 hr after injection of thiosulphate (4)	Percent change from preceding value (5)
0.90	0.80	-11	1.6	100.0
1.25	1.75	+40	2.8	60.0
1.00	1.35	+35	1.2	-7.7
0.50	0.30	-40	3.0	900.0
1.50	0.80	-46	1.2	50.0
1.00	1.80	+80	0.8	-55.0
0.40	0.70	+75	1.6	128.0
1.40	0.60	-57	1.4	133.0
0.8	-	-	0.8	Not available
2.00	0.60	-70	0.7	16.0
1.80	0.80	-55	1.2	50.0
0.50	1.20	+140	0.5	-58
1.00	1.40	+40	1.6	15.0
0.80	1.00	+20	1.7	70.0
0.80	1.00	+20	1.0	-
-	0.70	-	3.15	320.0
-	0.35	-	0.82	157.0

Increase of 50% or more 3/13 10/14 Fishers exact test P=0.031

Table 2. Level of thiocyanate expressed as potasium thiocyanate in mg% in urine 1 hr after injection of thiosulphate. A: 1 hr after 1st injection of thiosulphate, B: 1 hr after 2nd injection of thiosulphate and C: 1 hr after 3rd injection of thiosulphate

Code No.	A	B	C
352	1.45	2.2	2.4
354	1.15	1.4	2.2
356	2.65	4.55	5.0
357	0.6	0.6	2.6
403	2.1	0.5	2.1
411	0.7	-	0.9
415	2.0	1.4	0.8
417	0.5	1.0	0.5
421	-	0.8	1.6
424	3.1	-	4.4
426	1	2.3	1.1
428	0.5	1.6	1.0
430	1.4	0.6	0.3
433	0.4	0.9	0.4
436	1.1	1.3	0.8
438	1.7	1.0	0.7
439	0.8	1.5	0.8
440	10.5	10.5	4.7

Table 3. Change in the level of thiocyanate in mg% as potasium thiocyanate in urine after an injection of glucose only

Code No.	1 hr after glucose injection	1 hr after 2 injection of glucose	% increase or decrease over the preceding value
435	0.9	0.8	-11
351	1.25	1.75	+40
353	1.0	1.35	+35
355	0.5	0.3	-40
402	1.5	0.8	-46
405	1.0	1.8	+80
410	0.4	0.7	+75
412	1.4	0.6	-57
416	0.8	-	-
437	2.0	0.6	-70
420	1.8	-	-
422	0.5	1.2	+140
425	1.0	1.4	+40
427	0.8	1.0	+20
429	0.8	1.0	+20

Table 4. Level of thiocyanate in urine of 5 individuals living in Bhopal after Bhopal Gas Tragedy. Thiocyanate levels were expressed as potasium thiocyanate in mg%

Smoker	Non-Smoker	Before injection of thiosulphate	1 hr after injection	% change	1 hr after 2nd injection of thiosulphate	1 hr after 3rd injection of thiosulphate
-	√	2.1 (1.65)	2.4	+15	1.8	1.8
√	-	2.3 (1.9)	2	-12	2.2	3.2
-	√	0.5 (0.35)	0.35	-30	1.2	0.6
-	√	0.85	0.95	+10	0.75	1.2
-	√	1.15	0.7	-40	0.7	0.7

Table 5. Relationship of thiocyanate in urine to the exposure of people to the gas tragedy directly or indirectly through the contact of the gas victims in the hospital

Nature of Group	Size of the group	% people having 1 mg%or more as potasium thiocyante in urine
People exposed to various extents directly to gas leak on 2nd December, 1984	48	65
Hospital workers exposed to gas victims	10	70
People living in areas of Bhopal unaffected by the gas leak	30	33

October 5, 1988

To

The Hon'ble Supreme Court,

New Delhi (INDIA)

Most respectfully I wish to submit the Hon'ble Supreme Court the following:-

1. I am the Chairman of the Supreme Court Committee appointed by the Hon'ble 'Court to look in to the several matters arising 'out of Nishit Vohra Petition. I also happen to be one of the main persons of the Toxicology Research Group of the Scientific Investigations in the Bhopal Gas tragedy apart from my being the Additional Director General, ICMR and the Director, Institute of Pathology. Both myself and Prof. Heeresh Chandra, another member directly appointed by the Supreme Court are concerned with the autopsy studies and scientific aspects of Toxicology. Both of us are responsible for the basic scientific contributions on Urinary Thiocyanate studies on demonstration of binding of MIC or Carbamoylation of Hemoglobin and tissue proteins two very significant findings in the Bhopal Disaster. These findings arc unique to the Bhopal Gas Disaster and are not at all reported in any of the literature on MIC toxicity in the earlier studies of Union Carbide as well as the subsequent work done in U.S.A. Even the work done with MIC on experimental animals by us in DRDE, Gwalior has shown Carbamoylation of Hemoglobin in but not the alteration Urinary Thiocyanate or response to Sodium Thiosulphate. I shall refer to it a little later.

2. The original petition submitted before the Court was related to the wider use of Sodium Thiosulphate as a detoxifying therapeutic agent in the gas victims.

   In this context the Hon'ble Supreme Court was pleased to constitute a Committee to look into this issue as the primary responsibility of the Committee In addition there were other is such as epidemiology and long term effects. I as the Chairman of the Committee and all of us in the Committee addressed ourselves to the primary question of Sodium Thiosulphate therapy its continuing efficacy distribution of the drug' by the Govt. and above all the question as to how long this type of treatment would be necessary. I may be permitted to point out that as per the Supreme Court Order, the opinion of the committee appointed by the Hon'ble Supreme Court shall be conclusive and binding and not questioned.

3. Soon after the Committee went into these questions, it became apparent that the height of the demand for Sodium Thiosulphate was declining both as judged by the response of the patients as well as the declining trends of baseline of post injection of Urinary Thiocyanate values.

4. Accordingly, by the middle of 1986 itself, we decided to submit to the Hon'ble Court our recommendations in this regard. However, Dr. Anil Sadgopal and Mr. Surjit K, Das persisted that the Committee should address itself to other tasks. But the rest of the other five technically competent medical experts felt that having discharged our duty on Sodium Thiosulphate therapy, we should seek the guidance or the Supreme Court in. the matter of terms of reference. This is due to our very genuine opinion that the other tasks are being adequately taken care of by the State and Central Governmental agencies and scientific organizations. We also felt that duplication of such efforts by the Supreme Court Committee may not be desirable. Hence, we sought the guidance of Supreme Court as submitted in our report from 29.12.1987 onwards.

5. Although the meetings in general were rough and boisterous and continuously disturbed both within and outside we tried to finalize the opinion of the Committee in the meeting by the middle of 1986 and definitely at the meeting of 13th and l4th December, 1986. The report of the Majority Croup was ready by the end of December 1986 itself. But the Minority members wanted to submit details of their report. Hence, it was decided to await their reports.

6. Subsequently the. Minority Group had submitted their report .to the Supreme Court towards the end of October 1987 which is before the Court. More importantly, contrary to the decision right from the beginning of not giving alarming press statements the Minority Group had resorted to systematic campaign of misinformation in the Press.

7. Therefore, without further awaiting the reports of the Minority Group we members of the Majority Group submitted

our report on 29th Dec, 1987. We are awaiting the guidance of the Supreme Court in this regard as reflected in all our subsequent submissions before the Hon'ble Supreme Court.

8. In the meanwhile, as per our very genuine fears and anxiety we find the minority members indulging in their methods of misinformation. To site an example I wish to submit the question and answer type of press interviews by Dr. A. Sadgopal in the leading magazines like the Frontline of THE HINDU. Therein they have falsely attributed the genuine scientific work postulated and irritated by me on tissue Carbamoylation to Dr. M. G Karmarkar. Our attention to this was drawn by the Chairman of the Scientific Commission for Bhopal Gas Leak. We addressed a polite letter to Dr. Karmarkar and the responses there too are also being submitted as an illustration of wrong information that the minority members are circulating.

9. In this context I as a Chairman of the Supreme Court Committee wish to add that we were alarmed at the spate of wrong statements being made even before the Hon'ble Court Supreme Court should give their guidance and decision in this matter. Therefore, we members of the Majority Groups recently met and decided to submitted the Hon'ble Supreme Court the statements being made by the Minority Group and detailes justifying our stand that there is no need for generalized use of Sodium Thiosulphate therapy at this present juncture in view of the declining trends on urinary Thiocyanate levels and at best limited use of Sodium Thiosulphate in any particular case could be considered.

10. At the outset, I wish to point out that there is a lot of confusing about the very premise of continuing circulating toxins advocated by the Hon'ble Minority members. The immediate or acute damage and after effects caused by poisoning (not toxins) are totally separate issues and must be distinguished. The former may be treatable by antidotes which may neutralize the chemical poison. At the same time it may have no effect on the permanent sequaelae of tissue and organ damage. The facts that patients continue to be ill does not justify indiscriminate use of the earlier antidote. However, effective it might have been earlier. Other modalities of treatment symptomatic or otherwise have to be instituted as is being done in Bhopal. Such indiscriminate use as is being advocated by the minority members will attract scientific ridicule and would be unethical and against the elementary norms of medical ethics. The Hon'ble Court may kindly ponder over the issue of the possible consequences, risks and dangers involved. As it is, according to UCC, there is no known antidote to MIC. It did suggest use of sodium thiosulphate if cyanide poisoning is suspected, thereby implying such a possibility. But this suggestion early in December 84 was withdrawn in indecent haste by the Union Carbide through the official channels. A vast majority of local, national, transnational and international experts were against use of sodium thiosulphate.

11. Some of us dared to pursue the matter successfully in the teeth of opposition. We had even managed to develop a test for monitoring the efficacy of sodium thiosulphate treatment by estimating the urinary thiocyanate levels. This was done in the teeth of opposition. That is the reason and basis for carrying out the Double Blind studies at every stage. Firstly we had to establish the efficacy of the NaTS treatment and secondly we had to ensure the need for its continuing use. Surely, nobody can prescribe or improse any treatment which is no longer warranted. It is a matter for satisfaction that this very delicate issue had been handled safely in the aftermath of a great disaster. It is not possible for anyone to vouchsafe the implicit hazards of an unimaginable campaign of detoxification of an entire city. In not all cases it is possible to have full 'recovery' after exposure to a poison. Long after the poison is neutralized by natural process or by an antidote, the consequences of the damage will remain. We are not dealing with acute diseases like cholera or gastroenteritis, nor are we dealing with disease like tuberculosis or leprosy. The very philosophy of the Geneva convention banning chemical warfare is because of the inherent difficulties of treating the permanent damage. In the unfortunate situation created by Bhopal, every effort is being made to meet the challenges of medical care and management.

12. However, in view of the persisting controversy that is being perpetuated by the members of the Minority Group on Continuing Circulating Toxins' advocating the indiscriminate use of NaTS, I wish to make the following:-

13. Early in the wake of the disaster of Methyl Isocyanate gas leak in Bhopal there were strong suspicions about the possibilities of death being caused by Hydrogen cyanide (HCN). This was based on the autopsy studies with characteristic cherry red colour of the blood and internal viscera like the lung and the brain.

14. These impressions of Prof. Heeresh Chandra was further reinforced by some preliminary information produced by Dr. Daundrer, a German Toxicologist who had visited Bhopal and carried out some laboratory tests on the blood

samples of survivors. There was suggitive evidence of the of the presence of the cyanide as per the chemical analysis of the CBI Toxicologist Mr. Besaria.

In fact, even the Union Carbide in its early messages suggested that in case cyanide poisoning was suspected. Sodium thiosulphate injections could be given in the standard manner i.e. along with sodium nitrite. However, this message was withdrawn through the official channels.

15. Even the postmortems after a lapse of 10-12 days continued to show similar gross features of cherry red discoloration. It was at that stage that the team from the Institute of Pathology led by myself, Dr. S Srirarnachari, Dr. HMK Saxena and Dr. B Das Gupta were associated with the post-mortem studies and also with the toxicological investigations which were being pursued vigorously in the Medico-legal Institute under Prof. Heeresh Chandra's guidance and direct supervision.

16. The several possible causes for the reddish discoloration of the blood and viscera in the context of the Bhopal Gas Disaster were critically examined even in the midst of all the urgency and lack of information and prevailing confusion. Direct vision spectroscopy and spectrophotometry of the blood did not reveal any evidence of Carboxy–hemoglobin nor was there any evidence suggestive of Meth–hemoglobin on samples of blood collected from the dead bodies in the Mortuary. Instead, standard bands suggestive of Oxy-hemoglobin were observed. These findings have a great bearing on the case. We shall not deliberate further. These findings lent further support to the possible role of hydrogen cyanide (HCN) being responsible for the gross discoloration of the organs. Just as in the case of all discoveries, wisdom dawns suddenly but it takes time. We in the Toxicological team were yet to make our new and original suggestion that when hemoglobin reacts with MIC through the process of 'Carbamoylation' an equally intense red colour would develop. Unfortunately we are forced to give these explanations at a premature stage of the trial.

17. In parenthesis it may be added that such a possibility was postulated by me on 29th Dec. 1984, This was soon followed 'up by me with the help of Dr. A. Ramaiah of the AIIMS and not Dr. M. G. Karmarkar as falsely and erroneously propogated by Dr. Anil Sadgopal in his press releases, and denied in writing by Dr. Karmarkar that he is not connected even remotely with carbamoylation studies.

18. Even prior to the postulation of carbamoylation of blood (binding of hemoglobin with MIC), as early as 18th of December 1984, while carrying out autopsy I Dr. Sriramachari, suggested that it might be worthwhile examining the samples of urine of the dead bodies for the presence of elevated levels of urinary thiocyanate since sufficient time would have elapsed for the Hydrogen cyanide to be detoxified or neutralized by the natural enzymatic systems such as Rhodanese of the liver. It is also felt that apart from the contributory role of HCN in some of the initial deaths, there could be a unique toxicity due to the release of cyanide radical by its reactants which might have been present in the poisonous gases that had formed during the gas leak. A paper on the pyrolysis of MIC published by Blake and Izadi Maghsoodi in 1982 from U.K. which refers to break down products such as CO, HCN and more interestingly additive compounds of MIC and HCN and carbo-di-amides have virtually been ignored.

Some of them by their slow release of cyanide radicals could contribute to the known toxic compounds such as MIC inhaled by the exposed population. Right from the very beginning we reiterated the possible role of slow reactions.

19. The preliminary findings of impaired Hemoglobin were confirmed by a more elegant method of investigation such as Gas Chromatography. This unique work was accomplished by us in the DRDE, Gwalior with the help of Dr. P. K. Ramachandran and his colleagues. There was hardly any information on the subject, both from UCC side and available world opinion. There were credulous questions from some of the scientists in the United Kingdom. Rightly we suspected that such evidence any of binding of MIC with the Hemoglobin of blood may not be present beyond the normal life span of 120 days of red-blood cells. We pursued the matter vigorously and collected the relevant information by March 1985.

20. Knowing fully well the hazards of such new knowledge being discarded or ignored by other parties. We were careful enough to await our own studies on experimental animals since there was no precedent. We are happy to inform you that we did succeed and have established that MIC enters the body, gets into the blood stream and combines with hemoglobin. Subsequently Prof. Heeresh Chandra, and his colleagues not only confirmed the

findings on Human Blood samples of Bhopal Gas Victims, but established that MIC is lodged in the tissue proteins of different organs like the liver and lung in the autopsy specimens right upto the middle of 1986. This is a great achievement of Indian Science.

21. All the millions of Dollars spent in the post Bhopal Disaster carried out in USA is silent on this vital issue of the presence of *hemoglobin* and tissue proteins of at least MIC. Dr. Bucher's remarks on this subject are revealing, yet our friends expect from UCC a Package of information on the toxicity of all the compounds likely to have been released. The learned Counsel of UCC has promised to provide this already available negative information published by Alarie, et al., on page 166 under the heading "cyanide like effect". In fact it was postulated by me that unlike the classical "cyanide pool" hypothesis illustrated in R. T. Williams Book on Detoxification Mechanisms (1959), it might be necessary to visualize the possibility of toxic chemicals capable of releasing slowly cyanide radicals. For this purpose right from December 1984, a working hypothesis of a possible "cyanogen pool" to be distinguished from, "cyanide pool" was clearly enunciated. A working hypothesis still gives room for any other possible mechanism. Alternate hypotheses such as MIC contributing to thiocyanate under the influence of Rhodanese were postulated by people like M. G. Karmarkar, on whom our friend Dr. Sadgopal is relying inspite of its absurdity.

22. It is in the course of the earliar vigorous, discussions that I, Dr. Sriramachari suggested that it might be worthwhile autopsy samples of urine where available are examined for urinary thiocyanate levels. It was also, felt that such an estimation in the urine of surviving patients, if found elevated, may have some diagnostic significance. More importantly we thought it may even be used to assess the therapeutic response or efficacy of sodium thiosulphate which was already being used in Bhopal from the middle of December 1984. A series of investigations were carried out in the Medico-Legal Institute and the work was initiated as early as 20th or 21st of December 1984. There were some positive indications but the method needed quantification. Since this is not a routine investigation the details of the method employed for estimation of thiocyanate concentrations in biological fluids was obtained from the NIN, Hyderabad where some related studies were carried out way back in 1954.

23. It was also known that thiocyanate estimations were being done in the lnstitute of Nuclear Medicine & Allied Sciences (INMAS) as part of their investigation on thyroid function. Hence, Brig. Lakshmipati then Director of the Institute of INMAS, was contacted and requested to carry out the estimations on samples of urine of Bhopal gas victims. Since INMAS was normally carrying out SCN estimations in serum (and not on urine) it was agreed that they would carry out a preliminary study on normal controls in Delhi (i.e. within their Institute).

    It was found that the normal values ranged from 0-0.5 mg% whereas in 14 out of 54 samples the levels were above 0.5 mg%. In 5 out of these 14 samples the values were more than 1 mg% and the rest between 0.5-1 mg%. Hence, as an immediate measure it was decided to take a figure of double the maximum of the normal values obtained as a cut off point for monitoring urinary thiocyanate studies in the Bhopal gas victims.

24. Simultaneously, steps were taken to generate data on normal urinary levels in unaffected areas of Bhopal and nearby places like Hoshangabad, Sehore etc. It must be pointed out that even in the midst of prevailing confusion and controversies and powerful forces pitted against the use of Sodium Thiosulphate as a detoxifying agent, this work was successfully initiated both for determining the pre- and the post-injection levels with a view to assess the response to Sodium Thiosulphate injections. A large number of patients showed 3-4 fold increase in the urinary SCN levels as compared to the upper limit of 0.5 mg.%. Furthermore, it was interesting that immediately after the administration of Sodium Thiosulphate injections, there was a further increase in the urinary thiocyanate levels. This indicated that the body's own mechanism on handling cyanide like materials was largely augmented by the administration of Sodium Thiosulphate as part of the "Sulphane pool". It is noteworthy that both on practical as well as theoretical considerations simultaneous injection of sodium nitrite as recommended for cyanide poisoning was not followed. Yet there was prompt and efficient mopping up of the circulating cyanide radicals.

25. Had it been a routine biochemical test, it would not have drawn the attention that urinary thiocyanate estimations have done. In those early days the response to sodium thiosulphate was so dramatic, that patients who were in a helpless situation were suddenly relieved of the major symptoms. All this has been documented as is well known. In retrospect, for the purpose of scientific study of the toxicological aspects of the Bhopal tragedy and also with a view to establish certain baseline scientific information for the rationale of Sodium Thiosulphate therapy and the

need for its continued use the thiocynate test had become a very valuable tool.

26. But the most important role of urinary thiocyanate estimations at that stage was to provide convincing evidence in the midst of violent and diamatically opposed views on the use of Sodium Thiosulphate injections. Hence, the ICMR undertook the first Double Blind Study towards the end of January, 1985. There was clear-cut statistically significant evidence that concomitant with clinical improvement there was marked elevation of urinary thiocyanate following the administration of Sodium Thiosulphate injections. These findings were statistically significant. This evidence constitutes the bedrock for the use of Sodium Thiosulphate and also a guideline for its subsequent use later as per the Press Release dated 12.2.1985. Hence the ICMR through its Toxicological teams, pursued the problem vigorously ever since. It was neither swayed by determined opposition to the use of Sodium Thiosulphate on the one hand, nor did it come into populistic demands for universal and large scale detoxification of the entire population. Instead the Toxicology team of the ICMR took a scientific and rational view of the whole matter. Certainly we are convinced of the efficacy of the Sodium Thiosulphate and pursued the problem and continued to monitor the need for its subsequent use by monitoring urinary thiocyanate levels.

27. It must be pointed out that the emphasis was on improvement of the symptomatology and the correlation with urinary thiocyanate levels. The pragmatic policy had really maintained the program of detoxification on an even keel without being buffeted by surcharged emotions on either side. It is regrettable that Dr. Anil Sadgopal and his friends had taken the latter course without any scientific justification. Nevertheless, the Toxicology team of the ICMR had continued their studies and have indicated the scientific basis for monitoring of samples of population at different period and in different places. It was never the intention to use this as a all time universal index or as a criterion for establishing the entire toxicological phenomena in the Bhopal Gas Disaster. It would appear that Dr. Anil Sadgopal and his friends have tried to capitalize on the true and genuine scientific investigations initiated by Dr. Heeresh Chandra and Dr. Sriramachari and carry it to absurd ends and virtually undermined it may be pointed out that during the last four years several organized studies had been undertaken apart from several thousands of patients over 15,000 whose urine hab been tested either before during or after the treatment with the course of Sodium Thiosulphate injections. The notable findings can be broadly classified into the following:

28. Double Blind Control Studies where patients are administered either one or more injections of Sodium Thiosulphate or a harmless control substance like glucose or normal saline. By and large such studies have been undertaken during the period January, 85 to March, 86 which includes the study last one being undertaken on the request and under the supervision of Dr. Anil Sadgopal who was then associate with the Jan Swasthya Kendra. The first Double Blind Study was undertaken in January, 1985. There were both subjective and objective improvements as well as a spurt of increased urinary thiocyanate level. It is on the basis of the first Double Blind Study, ICMR gave its recommendation of 12.2.85. With a view to enable large number of patients getting the injections, the ICMR in its recommendations of 4.4.1985 spelt out the guidelines. At that stage there was no need to prescribe or stiulate the limit of urinary thiocyanate level for eligibility of NaTS injections. There were persisting controversies in the medical circles to give or not to give the drug. Certainly ICMR can only lay down the guidelines but not impose itself to give or take injections.

29. Thereafter the Second Double Blind Study was undertaken in August-September 1985. Many of us felt that the response to the drug was still there. Unfortunately, some errors had crept in the control or placebo group and therefore the ICMR report had used the term “results inconclusive”. But it did not deter the continuing use NaTS.

30. The Third Double Blind Study was undertaken in the month of October-November, 1985. Here the procedure was modified with a view to assess the resultant benefit at the end of a course of injections. The interpretation varied. Some of us felt that the return to lower values in the, NaTS treated Group after the 3rd day onwards indicated that the circulating toxic material was flushed out, whereas they continued to be high in the control group. Although there was a declining trend, it was out of this careful consideration of the massive study of gas affected victims in Bhopal as against Hoshangabad and Sehore, that it was decided that the injections may be continued in symptomatic cases with elevated levels of urinary thiocyanate more than 1 mgm%. This level of 1 mgm% was based on the analysis of the exhaustive data collected by Dr. Heeresh Chandra and comparison of information available in Tobacco smokers in different parts of the World and in large scale population studies in Africa like Nigeria and Congo on people subsisting on Tapioca based diet.

31. We did not want to deny the drug to any patient needing it. At the same time we were cautious and careful to limit it to symptomatic cases with elevated levels over 1 mgm% of urinary thiocyanate as per the ICMR recommendations of 2nd Jan. 1986. The monitoring was continued.

32. In March, 1986, in association with Dr. Anil Sadgopal, both Prof. Heeresh Chandra and I conducted the Fourth Double Blind Study. This study was double checked by Dr. Sadgopal. In the TIFR the values were even lower if any. The results were indeed startling. The baseline values were low and the post-injection values were not raised. We informed Dr. Sadgopal and advised him that we should no longer flog a tired horse and should not run the risk of being unscientific and be a subject of ridicule, and that was the parting of the ways from July 86 meeting onwards.

33. *Pari passu,* we in the Toxicology group continued our studies on Hospital based and Community based gas exposees.

A. Clinical Studies

There were a large number of hospital-based studies undertaken both: in the Hamidia Hospital and in the 30 bedded Hospital since known as the Jawaharlal Nehru Hospital. In the latter, a large number of patients both children as well as adults have been followed up over a long period by Dr. P.S. Narayanan and myself.

A large number of community-based investigations on the gas affected population have been studied and analysed on a quarterly basis during the years 1985-86 and 1987. In addition, several hundreds of controls from the unaffected areas of Bhopal and from different batches of Army Jawans located in Bhopal were investigated for their urinary SCN levels. In all these studies several factors have been meticulously examined, taking into account smoking habits, consumption of vegetables rich in cyanogenic materials, etc.

The following distinct studies on urinary thiocynate were available for evaluation.

a) Study of 50 paediatric cases in the 30-bedded Hospital by Dr. P.S. Narayanan and Dr. Mullik. This is a retrospective analyses with clinical follow up and correlation of SCN values vis-à-vis relief recurrence and response to NaTS. It provides a three point follow up study ever a period of 6 months with progressively declining trends.

b) Dr. P.S: Narayanan's project on a cohort of 310 cases, from 1985 through 1988, Correlations of clinical, X-ray, PFT, Blood Gas and urinary SCN values were carried out in 285 individuals out of whom in 133 "two-point" data was availab1e. Secular trends in the former and poised test in the latter revealed very convincing information on the declining trends between 1985-86 and 1987-88.

c) Follow up studies on "therapeutic response" in 25 patients given two or more courses of NaTS injections in the 30-bedded Hospital in August 1985 through December 1985 showed a similar trend.

B. Community Based Studies

At the time of the 3rd Double Blind Study, a sizeable number of individuals were examined for urinary SCN levels in remote places like Hoshangabad and Sehore already referred to.

The massive data obtained in the Registers of the Medico-Legal Institute on urinary SCN values is available in terms of the normal and percentages of different levels of SCN and the overall "mean" at the end of each quarter over a three year period. Similar1y, the declining trend of the native above l mgm% over the 3 year period is available.

C. Study on 'Claim Cases'

At the instance of the Principal Secretary, Bhopal Gas Relief, a program was drawn up to recheck the values of individuals with high urinary SCN levels and see their response to NaTS injection by carrying out fresh baseline analysis followed by a post-injection estimation. This work was done in 1987 in over 130 patients or rather claimants. This information fully confirmed the current status of urinary SCN values as well as NaTS therapy, which was practically nil. Lastly, all these studies which have been carried over a long period of time have been compared and contrasted with the initial values both in terms of the urinary thiocyanate levels and the response to injection of Sodium Thiosulphate.

34. The bulk of these studies have revealed that there is an elevation in the urinary thiocyanate levels in the gas affected population and that there was a prompt response to sodium thiosulphate. However, by the end of 1985 and the first quarter of 1986 the values had tended to be normal and the response was poor. This is borne out by the sequential information that had been collected over representative samples as already stated. Hence, it is felt that in view of the declining trends, the need for Sodium Thiosulphate injections should be progressively tapered off taking due precaution that no patient with clinical symptoms and with elevated thiocyanate levels would be denied the drug. Fortunately the study of the claim cases and follow-up of some of the clinical cohorts had provided valuable information not only as to the declining trends and reduced or absent response to Sodium Thiosulphate but also the return to nearly normal levels.

35. It is particularly noteworthy that in all these investigations the figure of 1 mgm% has been repeatedly shown to be the cut off point vis-à-vis symptomatology.

36. The members of the Majority Committee were convinced of the above findings and therefore advocated the progressive withdrawal of Sodium Thiosulphate injections from March, 1986 onwards. Although Dr. Anil Sadgopal and Dr. Sujit Das had no valid scientific objections, they tried to perpetuate wrong ideas about the continued and unending use of thiosulphate for the entire affected population of Bhopal. The majority of the members refused to accept such an unscientific proposition and hence there has been a parting of the ways even on this major issue.

37. Independent of this, the Majority of the members felt that the purpose of the Committee set up by the Hon'ble Supreme Court of India had fulfilled its task after drawing valid conclusions on the continued use of Sodium Thiosulphate. Based on the experience with the interminable disputes and provocations of Dr. Anil Sadgopal and Dr. Sujit K. Das, the Group very sincerely feels that no useful purpose would be served by the Committee undertaking investigation of the other terms of reference. We are also of the unanimous opinion that these tasks are adequately taken care of by a wide range and variety of agencies and organizations both at the Central as well as State level. It was, therefore, felt that no useful purpose is likely to be served by parallel studies by the Supreme Court Committee and hence the majority members submitted in their report that the Hon'ble Supreme Court may reconsider the whole issue and give proper guidelines after taking into account all aspects of the matter.

38. It is, indeed, reprehensible that the Minority members who are by no means experienced in the field, have thought it fit to raise a sustained campaign against the Majority Group with a view to embarrass the Government and gain access to vital information which could be used for their own ends. The series of Press Reports and distortions of scientific facts and wild speculations contained in their reports and their publicity and propaganda through the media, speak for themselves. The Majority members arc quite concerned and apprehensive that such misuse of the role by the Minority members might adversely affect the legal proceedings of the Bhopal Gas victims, apart from the question of Sodium Thiosulphate being a "non-issue" ever since the middle of 1986 & more so at this stage.

39. Your Lordship the foregoing is not only my personal opinion but the unanimous opinion of 5 members, branded by the Minority as "Govt. servants, with no apparent concern for humanity". We highly object and pray your Lordship to ignore the untenable and unscientific observation and demands of the minority or upheld the considered opinion of the majority members.

40. May I also submit that according to the Hon'ble Court order, the opinion of the Supreme Court Committee is final and binding. Therefore, the minority members should not be allowed any more to indulge in irrelevant and untenable observations, which tend to subvert the opinion of the majority members who are 'subject specialists' in their respective fields.

Dr. S. Sriramachari

Chairman

Supreme Court Committee

CHAPTER

7 BLOOD & TISSUE CARBAMOYLATION

RIGHT from the very beginning of the autopsy studies of Bhopal Gas Disaster, Heeresh Chandra was guided by the 'intense congestion and unusual redness' of the LUNG and other target organs and especially the 'Cherry-Red Discoloration' of even the venous blood. He postulated that "Death in Bhopal Gas Disaster was due to Cyto-toxic & Histo-toxic Anoxia due to Irrespirable Gases". To understand the phenomenon of such a large-scale involvement of human beings and cattle alike, a series of investigations were carried out by the Pathology & Toxicology Team of the ICMR, under the joint leadership of Heeresh Chandra, Sriramachari and their associates.

Even at a very early stage, i.e., December 84, Sriramachari suggested another hypothesis for reddish discoloration of blood and viscera due to 'MIC-blocked Hb'. Keeping in view that the condition might disappear after one complete turnover of the entire blood within 120 days, strenuous efforts were made to clinch the issue, as early as possible. As a first step towards such a hypothesis, efforts were made to determine the reduction in the 'Free Amino Groups' of the blood of Gas Victims. This work was done at AIIMS, New Delhi, with the active association of Ramaiah and Roman Reddy. The reduced level of the end-terminal 'Free Amino Groups of Blood 'was estimated by the TNBS (Tri-Nitro Benzene Sulphonic acid) method of Robert Fields (1972); as compared with Controls, a reduction of 25 to 40 percent was demonstrated in the Bhopal Gas Victims. A brief account of the methodology and the results of this pioneering Clinical Laboratory study by Ramaiah, Reddy & Sriramachari is presented in the Report as ***Annexure 7.1***.

Carbamoylation Studies on Clinical Blood Samples

But soon after the Bhopal Gas Disaster, the UCC circulated an erroneous notion that before crossing aqueous surfaces of the 'alveolar-capillary barrier', MIC breaks down into relatively innocuous / non-toxic 'Methyl Amine & Di-Methyl Urea'. Contrary to their message, our preliminary studies established *prima-facie validity* to the additional hypothesis of Sriramachari that MIC binds to the Free End-Terminal Amino Acids of Hb and other constituents of the blood stream of the Bhopal Gas Victims. Based on the positive preliminary findings of Blood Gas Analysis and compensatory mechanisms like '2-3 DPG levels', the possibility of N-Carbamoylation of the end-terminal Valine residues of Hb was pursued progressively by different techniques. The initial study of reduction of 'Free Terminal Amino Groups' by TNBS technique was followed by a positive demonstration of binding of MIC with a-amino group of terminal Valine residues of a- and/or b- chains of Hb. This later work was accomplished with the cooperation of Ramachandran & Associates at DRDE Gwalior, where facilities for Gas Chromatography (GC) were readily available even at that time. Before starting the GC studies, Carbamylated Valine standards for the investigation were freshly prepared, with both inorganic cyanate as well as Methyl Isocyanate, synthesized as per DRDE schedule. Samples of blood from Laboratory rats & rabbits were successfully shown to be N-Carbamoylated, both *in-vitro* as well as *in-vivo*. The GC characterization of Methyl Valine Hydantoin (MVH) was established and also published in 'Journal of Chromatography (1988)' **(*Annexure 7.2*).**

This was followed by collection of 'Clinical Blood Samples' from the Toxicology Project entitled "Pulmonary Function Tests and Blood Gas Analysis", under Narayanan & Sriramachari, in close association with Heeresh Chandra. Varying grades of MVH +vity, ranging from 1+ to 3+, were demonstrated in a total of 20 out of 160 samples which were collected during the 1st Quarter following the Disaster. While the details of the investigation are given as ***Annexure 7.3***, the summary is shown below in Table 7.1. By contrast, autopsy samples of whole blood as well as from the viscera showed a greater positivity (69/291) as well as the extent or the degree of Carbamoylation, a majority of which belonged to the 1984 autopsies.

Table 7.1. Blood Samples analysed by GC for Carbamoylation of Hb (DRDE, 1990).

Number of Samples	Carbamoylation test by GC
4	+++
4	++
12	+
140	Negative
Total 160	Positive = 20

The successful demonstration of MIC-based MVH in both Experimental & Clinical samples is a noteworthy new finding. However, unlike the results of 'autopsy studies', the percentage of positivity as well as the concentrations of the 'Valine Hydantoin' levels were not as high as per the expectations. Possibly, the 'low incidence' was a reflection of lower levels of exposure amongst 'survivors' and a normal turnover of erythrocytes. In spite of several efforts, 'Paired Blood Samples' from the survivors could not be analysed confirm further lowering of N-Carbamoylation levels.

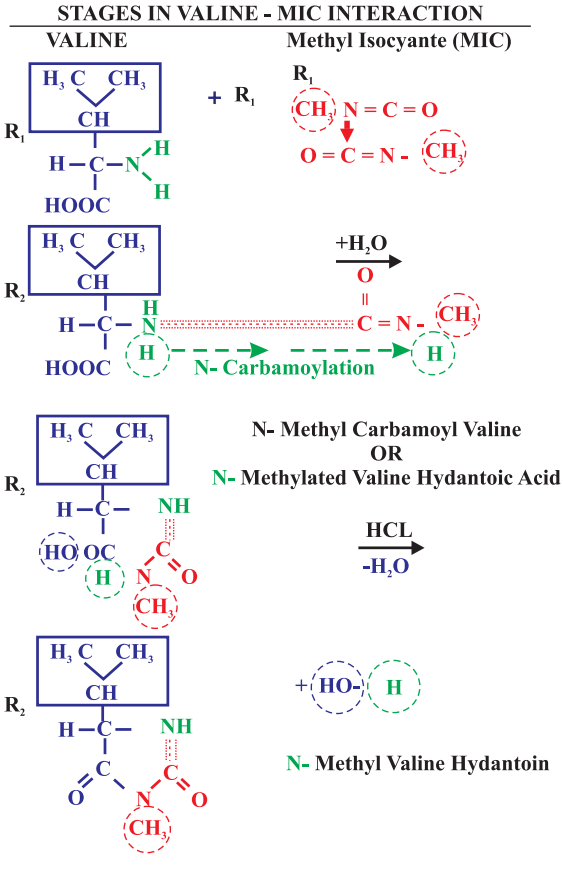
Study of Autopsy Blood & Tissue Samples for Carbamoylation

Development of Methodology: Early in the wake of TNBS & MVH studies, Clinical Samples of Bhopal Gas Victims were positive for binding with MIC. So, Heeresh Chandra quickly decided to extend the Carbamoylation Studies to include other end-terminal amino-acids of tissue-proteins as well in the 'autopsy material', carefully preserved in accordance with the guiding principles of study of each one of the autopsies related to BGD. In fact the severity of the Biochemical changes/lesions were expected be more severe in the fatal cases.

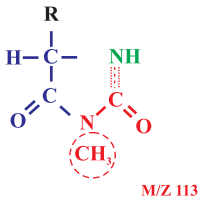
Preparation & Confirmation of Standards: The steps of preparation of volatile N-Carbamyl hydantoin derivative of a typical amino acid like Valine, as suggested by Sriramachari, was based on the method of Manning et al, 1973 and elaborated by Ramachandran et al. 1988 as per the following chemical reaction:

$$CH_3NCO + RCHNH_2.COOH \rightarrow CH_3NHCONHRCHCOOH.$$

Synthesis of Hydantoin Derivatives of Amino Acids: Purified samples of all the 21 Amino Acids at 4 mmol concentrations in 20 ml of water were taken separately, and 8 mmol of methyl isocyanate was added and the mixture was stored for 18 h at 50°C. The pH was maintained at 8.1 with 1 N acetic acid for neutralization with Remi magnetic stirrer. The reaction was stopped by addition of 0.7 ml concentrated HCl. The solution was heated at 100°C for 60 min for formation of corresponding Hydantoins of amino acid with MIC. The solution was evaporated to dryness at 45°C under reduced pressure and the residue was resuspended in 3-4 ml water and filtered. The white precipitate was dried over-night over NaOH pellets in a vacuum dessicator. The product was analysed for formation of hydantoin derivatives. But out of the 21 amino acids investigated, only the following eleven were successfully converted into corresponding volatile methylated hydantoin derivatives:-



Chemical pathways of N-carbamoylation



(HYDANTOIN FRAGMENT)

1. Glycine (MW 114),
2. Alanine (MW 128),
3. Serine (MW 144),
4. Valine (MW 156)
5. Threonine (MW 159),
6. Cysteine (MW 160),
7. Hydroxyproline (MW 170),
8. Glutamine (MW 185),
9. Glutamic Acid (MW 185),
10. Methionine (MW 188)
11. Phenylalanine (MW 204).

The crystallized compounds were analysed on GC-ITD. The fragment m/z 114 represents the cyclic hydantoin component. The MVH was eluting m/z 113 at 5.56 min. The principal mass fragments of this reference compound were m/z 114, 57, 157, 56, 55, 115 and 59 in decreasing order of intensity. The total mass spectrum was used for a library match. Apart from synthesised MIC compound, another reference standard was also obtained from DRDE, Gwalior, and a comparison was made. The melting point in both the standards was observed to be 210- 211ºC. The ultraviolet absorption spectrum as presented gave an absorption maxima at 233 nm in the range of 200 to 400 nm and the insert shows mass fragmentation pattern of the Carbamylated Amino Acids including the Cyclic Hydantoin constituent.

MATERIALS AND METHODS: In the Toxicology Project at MLI, N-Carbamoylation studies were undertaken by HPLC, GC and GC-MS methods with ITD system. As detailed elsewhere, the autopsies of gas-exposed victims were performed at the MLI, Bhopal ever since 3rd December, 1984. Simultaneously, a fairly large number of Clinical Blood Samples were also collected from several associated hospitals of MGMC, Bhopal from Sultania Zanana Hospital; other Clinical Samples like 'Placenta & Cord Blood' were also included from each Autopsy, approximately 5-50 ml of Blood and 10-50 gms of 'Fresh Tissue' from organs like Brain, Lung, Heart, Liver, Kidney & Spleen were collected and stored in glass vials, bottles or plastic bags and preserved in 'deep freezers'. Test and Control samples were drawn at random, as allowed for the study, from the sample stock and classified area-wise, based on the individual case history. All the samples were labeled and registered according to the routine procedure of the MLI. Use of preservatives for the samples was purposely avoided. Table 7.2 shows collection of different samples ever since 3rd December 1984 till December 1990. GC-MS studies were conducted on 207 exposed Clinical as well as Autopsy cases. Out of a total of 351 tissue samples of 1984-90 which were analysed, 70 samples were of Clinical cases.

Table 7.2. Distribution of 429 Clinical & Autopsy Samples of various Body tissues of 236 cases taken for GC-MS analysis

Tissue	1984		1985		1986 - 1990		Total	
	E	C	E	C	E	C	E	C
Blood	2	-	30	-	20	10	52	10
Placenta	-	-	4	-	-	-	4	-
Cord	-	-	4	-	-	-	4	-
Total	2	-	38	-	20	-	60	10
Blood	61	2	28	2	35	12	124	16
Brain	9	1	5	1	6	2	20	4
Lung	14	1	10	3	22	9	46	13
Liver	10	1	6	2	19	8	35	11
Heart	8	1	3	2	14	6	25	9
Kidney	5	-	3	2	10	8	18	10
Spleen	9	-	5	1	9	7	23	8
Total	116	6	60	13	135	12	291	71
Grand Total	**118**	**6**	**98**	**13**	**155**	**12**	**351**	**81**

E = Exposed Area C = Control Area

Preparation from Cryo-preserved Samples

In each case, 0.5 to 2.0 ml blood sample was taken and the protein was precipitated by adding 2% HCl in cold acetone. The precipitated protein was washed several times with cold acetone till it was free from Haem and finally

washed with cold diethyl ether. The washed protein precipitate was dried under reduced pressure over warm water. The dried white powder of blood protein was suspended in 1.0 ml of 50% acetic acid and heated at 100ºC for one h after adding equal volume of concentrated HCl for cyclization of N-Carbamylated amino acid. After cyclization, the reaction tube was cooled in ice and 10 N NaOH was added till solution attained a pH between 3-5, followed by addition of about 1 ml saturated NaCl solution. Ethyl acetate was then added to the hydrolysate and Methyl-Hydantoins formed were extracted by mixing the contents of the tubes for two minute on vortex mixer. The solvent was stripped off under reduced pressure and the residue was desiccated over silica gel over-night (Sriramachari et al. 1991).

Standardisation of Method for Sample Analysis

HPLC of the hydantoins was carried out according to the method of Sharma et al, (1990). The hydantoins were subjected to chromatography on Zorbax ODS column (4.6 mm x 25 cm), with the mobile phase of water-methanol (80:20) at a flow rate of 1 ml per min. The detector wavelength was 210 nm. The detection limit of the method for Valine Methyl Hydantoin was in nano-grams. However, this method was found to be unsuitable for estimating other 'Hydantoin derivatives' such as, Glycine, Valine, Threonine, Methionine and Phenyl Alanine. Instead, for their estimation, GC-NPD and GC-ITD methods were found to be better for most of them. The dried residue was first dissolved in 200 µl ethyl acetate and 0.5 to 1.0 µl of this solution was injected into gas chromatograph configured with either Nitrogen Phosphorus Detector (GC-NPD) or Ion Trap Detector (GC-MS). GC-NPD analysis was carried out on Perkin-Elmer model Sigma 300 gas chromatograph glass column 6' x 1/4" OD x 0.5 mm ID packed with 3% EGSP-Z on 100/120 mesh gas chrom Q, nitrogen carrier gas 25 ml/min, Injector 210ºC, column 200ºC, detector 230ºC, NPD bead current 400, gas flow of hydrogen 30 ml/min and air 100 ml/min. Quantification and recording of the peaks were made using Perkin-Elmer Model LCI-100 laboratory computing integrator.

GC-MS studies were carried out on a Varian model 3400 Capillary Gas Chromatograph interfaced with Mass Spectrometer-Ion Trap Detector (ITD) model 800 of Finnegan MAT Ltd., UK. This system was used to obtain GC retention time data as well as mass spectral data for identification of the compound under study. Column DB-5, 0.25 um film thickness (J & W Scientific), helium linear velocity 16 cm/sec with column head pressure of 10 psi, oven 180ºC to 230ºC at 5ºC/min (180°C for 1 min, and 230ºC for 10 min), injector 250ºC, transfer line 250ºC, ITD Scan mode m/z range from 50 to 200 amu (1 scan/sec) with ion source temp of 216-220ºC and EI 70 eV were used for the experiments. The data system for the ITD was an IBM PC-XT with standard GC-MS software of Finnegan MAT for tuning, total ion chromatogram generation and library search routines.

Analysis of Exposed Tissue Samples for Methyl Carbamyl Derivatives as Hydantoins: From out of the preserved tissues of autopsy cases of exposed victims, which were collected between December 1984 and 1990; 150 cases were analysed for the presence of MIC-Carbamylated Amino Acids in terms of volatile Hydantoin derivatives. The details of number of cases analysed are presented in Table 7.2. By the end of 1989, the project acquired a much more sophisticated GC-MS with an Ion Trap Detector (Finnegan MAT, UK). As a result, MVH was demonstrated in a comparatively large series 38 out of 72 cryo-preserved autopsy blood samples. The fragmentation patterns of the reference or test samples of (exposed) victims were clearly characterized. MVH positivity was seen in 35/61 samples in the first month i.e., December 1984 and 9/24 in the next 9 months i.e., upto September 1985 (Table 7.3, 7.4 and 7.5). There was not much difference in the sex factor on carbamoylation, but a slight variation was observed in the elimination of carbamoylated amino acids in case of female group; rapid detoxification was observed in females as compared to male group.

Evidence of N-Carbamoylation in Autopsy Tissue Samples

The Lung tissues showed positive evidence of the presence of 7 out of the 11 N-Carbamylated amino acids, viz., Valine, Glycine, Alanine, Threonine, Methionine, Hydroxyproline & Phenyl Alanine, followed by five amino acids in Autopsy Blood Samples viz., Valine, Glycine, Alanine, Hydroxyproline, Phenyl Alanine, three amino acids in brain viz.. Valine, Glycine and Alanine, two each in Kidney, Spleen and Liver viz.. Valine and either Alanine in case of Spleen & Kidney and Threonine in case of liver; only Valine was Carbamoylated in case of heart tissue. However, individual samples varied in carbamoylation. The maximum number of several Carbamoylated amino acids of one tissue in case of lung was 6, blood 4, brain 3, kidney 2 and spleen 2.

Table 7.3. GC-MS Analysis of Postmortem Samples of Exposed Victims: Evidence of Carbamoylation and Tank Residue Compounds (1984 samples)

Date	Cases	Samples	Tissue	Analysed	Gly	Ala	MVH	Thr	Hpr	Met	Phe	MICT	DMI	2, 4 Dione	Spiro	m/z279
			Blood	33	5	4	19	-	-	-	-	15	8	1	3	4
			Brain	1	1	1	1	-	-	-	-	-	-	-	-	-
3.12.84	34	39	Lung	2	-	2	2	1	-	1	1	2	1	-	-	2
			Liver	1	-	-	1	1	-	1	1	-	-	-	-	-
			Heart	-	-	-	-	-	-	-	-	-	-	-	-	-
			Kidney	-	-	-	-	-	-	-	-	-	-	-	-	-
			Spleen	2	-	-	2	-	-	-	-	-	-	-	-	-
			Blood	16	3	5	11	-	1	-	1	10	3	-	1	2
			Brain	-	-	-	-	-	-	-	-	-	-	-	-	-
4.12.84	16	16	Lung	-	-	-	-	-	-	-	-	-	-	-	-	-
			Liver	-	-	-	-	-	-	-	-	-	-	-	-	-
			Heart	-	-	-	-	-	-	-	-	-	-	-	-	-
			Kidney	-	-	-	-	-	-	-	-	-	-	-	-	-
			Spleen	-	-	-	-	-	-	-	-	-	-	-	-	-
			Blood	12	1	1	5	-	-	-	-	2	1	-	-	-
			Brain	8	1	-	1	-	-	-	-	-	-	-	-	-
5.12.84	25	61	Lung	12	-	1	9	-	1	1	1	-	-	-	-	1
to			Liver	9	-	-	3	-	-	-	-	-	-	-	-	-
31.12.84			Heart	8	-	-	1	-	-	-	-	-	-	-	-	-
			Kidney	5	-	1	1	-	-	-	-	-	-	-	-	-
			Spleen	7	-	1	1	-	-	-	-	-	-	-	-	-
			Blood	61	9	10	35	-	1	-	1	27	12	1	4	6
			Brain	9	2	1	2	-	-	-	-	-	-	-	-	-
Total			Lung	14	-	3	11	1	1	2	2	4	1	-	-	3
Dec: 84			Liver	10	-	-	4	1	-	1	1	-	-	-	-	-
			Heart	8	-	-	1	-	-	-	-	-	-	-	-	-
			Kidney	5	-	1	1	-	-	-	-	-	-	-	-	-
			Spleen	9	-	1	3	-	-	-	-	-	-	-	-	-
Grand Total	**75**	**116**		**116**	**11**	**16**	**57**	**2**	**2**	**3**	**4**	**31**	**13**	**1**	**4**	**9**

Table 7.4. GC-MS Analysis of Postmortem Samples of Exposed Victims: Evidence of Carbamoylation and Tank Residue Compounds (1985 samples)

Date	Cases	Samples	Tissue	Analysed	Gly	Ala	MVH	Thr	Hpr	Met	Phe	MICT	DMI	2, 4 Dione	Spiro	m/z279
01.01.85 to 31.03.85	11	28	Blood	6	-	-	3	-	-	-	-	2	-	-	-	-
			Brain	4	-	1	1	-	-	-	-	-	-	-	-	-
			Lung	5	1	-	2	-	-	-	-	-	-	-	-	-
			Liver	4	-	-	-	-	-	-	-	-	-	-	-	-
			Heart	2	-	-	-	-	-	-	-	-	-	-	-	-
			Kidney	3	-	-	-	-	-	-	-	-	-	-	-	-
			Spleen	4	-	1	-	-	-	-	-	1	-	-	-	2
01.04.85 to 30.06.85	9	11	Blood	9	-	-	3	-	-	-	-	1	-	-	-	-
			Brain	-	-	-	-	-	-	-	-	-	-	-	-	-
			Lung	1	-	-	-	-	-	-	-	-	-	-	-	-
			Liver	1	-	-	-	-	-	-	-	-	-	-	-	-
			Heart	-	-	-	-	-	-	-	-	-	-	-	-	-
			Kidney	-	-	-	-	-	-	-	-	-	-	-	-	-
			Spleen	-	-	-	-	-	-	-	-	-	-	-	-	-
01.07.85 to 31.09.85	11	13	Blood	9	-	-	3	-	-	-	-	2	-	-	-	-
			Brain	-	-	-	-	-	-	-	-	-	-	-	-	-
			Lung	3	-	-	-	-	-	-	-	-	-	-	-	-
			Liver	1	-	-	-	-	-	-	-	-	-	-	-	-
			Heart	-	-	-	-	-	-	-	-	-	-	-	-	-
			Kidney	-	-	-	-	-	-	-	-	-	-	-	-	-
			Spleen	-	-	-	-	-	-	-	-	-	-	-	-	-
01.10.85 to 31.12.85	4	8	Blood	4	-	-	-	-	-	-	-	-	-	-	-	-
			Brain	1	-	-	-	-	-	-	-	-	-	-	-	-
			Lung	1	-	-	-	-	-	-	-	-	-	-	-	-
			Liver	-	-	-	-	-	-	-	-	-	-	-	-	-
			Heart	1	-	-	-	-	-	-	-	-	-	-	-	-
			Kidney	-	-	-	-	-	-	-	-	-	-	-	-	-
			Spleen	1	-	-	-	-	-	-	-	-	-	-	-	-
Total Dec 85	35	60	Blood	28	-	-	9	-	-	-	-	5	-	-	-	-
			Brain	5	-	1	1	-	-	-	-	-	-	-	-	-
			Lung	10	1	-	2	-	-	-	-	-	-	-	-	-
			Liver	6	-	-	-	-	-	-	-	-	-	-	-	-
			Heart	3	-	-	-	-	-	-	-	-	-	-	-	-
			Kidney	3	-	-	-	-	-	-	-	-	-	-	-	-
			Spleen	5	-	1	-	-	-	-	-	1	-	-	-	2
Grand Total	**35**	**60**		**60**	**1**	**2**	**12**	**-**	**-**	**-**	**-**	**6**	**-**	**-**	**-**	**3**

Table 7.5. GC-MS Analysis of Clinical Samples of Exposed Victims: Evidence of Carbamoylation and Tank Residue Compounds (1984-90 samples)

Date	Cases	Samples	Tissue	Analysed	Gly	Ala	MVH	Thr	Hpr	Met	Phe	MICT	DMI	2, 4 Dione	Spiro	m/z279
1984	2	2	Clinical Blood	2	-	-	1	-	-	-	-	-	2	-	-	-
1985	35	38	Clinical Blood	30	-	-	5	-	-	-	-	-	-	-	-	-
			Placenta	4	-	-	-	-	-	-	-	-	-	-	-	-
			Cord	4	-	-	-	-	-	-	-	-	-	-	-	-
1986 to1990	20	20	Clinical Blood	20	-	-	-	-	-	-	-	-	-	-	-	-
Total	57	60	Blood	52	-	-	6	-	-	-	-	-	2	-	-	-
1984 to			Placenta	4	-	-	-	-	-	-	-	-	-	-	-	-
1990			Cord	4	-	-	-	-	-	-	-	-	-	-	-	-

Clinical Samples: N-Carbamoylation of Hb & Presence of Tank Residue Components

Table 7.6. Autopsy Tissue Findings: N-Carbamoylation and Tank Residue Compounds

Date	Cases	Samples	Tissue	Analysed	Gly	Ala	MVH	Thr	Hpr	Met	Phe	MICT	DMI	2, 4 Dione	Spiro	m/z279
Dec: 84	75	116	Blood	61	9	10	35	-	1	-	1	27	12	1	4	6
			Brain	9	2	1	2	-	-	-	-	-	-	-	-	-
			Lung	14	-	3	11	1	1	2	2	4	1	-	-	3
			Liver	10	-	-	4	1	-	1	1	-	-	-	-	-
			Heart	8	-	-	1	-	-	-	-	-	-	-	-	-
			Kidney	5	-	1	1	-	-	-	-	-	-	-	-	-
			Spleen	9	-	1	3	-	-	-	-	-	-	-	-	-
Total	**75**	**116**		**116**	**11**	**16**	**57**	**2**	**2**	**3**	**4**	**31**	**13**	**1**	**4**	**9**
			Blood	6	-	-	3	-	-	-	-	2	-	-	-	-
			Brain	4	-	1	1	-	-	-	-	-	-	-	-	-
Jan 85	11	28	Lung	5	1	-	2	-	-	-	-	-	-	-	-	-
to			Liver	4	-	-	-	-	-	-	-	-	-	-	-	-
Mar 85			Heart	2	-	-	-	-	-	-	-	-	-	-	-	-
			Kidney	3	-	-	-	-	-	-	-	-	-	-	-	-
			Spleen	4	-	1	-	-	-	-	-	1	-	-	-	2
Total	**11**	**28**		**28**	**1**	**2**	**6**	**-**	**-**	**-**	**-**	**3**	**-**	**-**	**-**	**2**
			Blood	22	-	-	6	-	-	-	-	3	-	-	-	1
			Brain	1	-	-	-	-	-	-	-	-	-	-	-	-
April 85	24	32	Lung	5	-	-	-	-	-	-	-	-	-	-	-	-
to			Liver	2	-	-	-	-	-	-	-	-	-	-	-	-
Dec 85			Heart	1	-	-	-	-	-	-	-	-	-	-	-	-
			Kidney	-	-	-	-	-	-	-	-	-	-	-	-	-
			Spleen	1	-	-	-	-	-	-	-	-	-	-	-	-
Total	**24**	**32**		**32**	**-**	**-**	**6**	**-**	**-**	**-**	**-**	**3**	**-**	**-**	**-**	**1**
			Blood	35	-	-	-	-	-	-	-	-	-	-	-	-
			Brain	6	-	-	-	-	-	-	-	-	-	-	-	-
1986	40	115	Lung	22	-	-	-	-	-	-	-	-	-	-	-	-
to			Liver	19	-	-	-	-	-	-	-	-	-	-	-	-
1990			Heart	14	-	-	-	-	-	-	-	-	-	-	-	-
			Kidney	10	-	-	-	-	-	-	-	-	-	-	-	-
			Spleen	9	-	-	-	-	-	-	-	-	-	-	-	-
Total	**40**	**115**		**115**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Grand Total	**150**	**291**		**291**	**12**	**18**	**69**	**2**	**2**	**3**	**4**	**37**	**13**	**1**	**4**	**12**

Table 7.7. Summary of Clinical & Autopsy Findings: N-Carbamoylation and Tank Residue Compounds

Date	Cases	Samples	Tissue	Analysed	Gly	Ala	MVH*	Thr	Hpr	Met	Phe	MICT	DMI	2, 4 Dione	Spiro	m/z279
1984 to 1990	57	60	Clinical Blood	52	-	-	6	-	-	-	-	-	2	-	-	-
			Placenta	4	-	-	-	-	-	-	-	-	-	-	-	-
			Cord	4	-	-	-	-	-	-	-	-	-	-	-	-
Clinical Total	**57**	**60**		**60**	**-**	**-**	**6**	**-**	**-**	**-**	**-**	**-**	**2**	**-**	**-**	**-**
1984 to 1990	150	291	Blood	124	9	10	44	-	1	-	1	32	12	1	4	7
			Brain	20	2	2	3	-	-	-	-	-	-	-	-	-
			Lung	46	1	3	13	1	1	2	2	4	1	-	-	3
			Liver	35	-	-	4	1	-	1	1	-	-	-	-	-
			Heart	25	-	-	1	-	-	-	-	-	-	-	-	-
			Kidney	18	-	1	1	-	-	-	-	-	-	-	-	-
			Spleen	23	-	2	3	-	-	-	-	1	-	-	-	2
Autopsy Total	**150**	**291**		**291**	**12**	**18**	**69**	**2**	**2**	**3**	**4**	**37**	**13**	**1**	**4**	**12**
Grand Total	**207**	**351**		**351**	**12**	**18**	**75**	**2**	**2**	**3**	**4**	**37**	**15**	**1**	**4**	**12**

* = Methyl Valine Hydantoin of end-terminal Valine Residue of Hb.

Interestingly, all these samples were from December 1984 collection. It was observed that Alanine stood in the second order next to Valine in susceptibility of N-Carbamoylation. The blood samples which were positive for Carbamoylation, were all positive for Valine Carbamoylation, i.e., all the 44 cases were positive for Valine. However, Glycine and Alanine Carbamoylation was seen in blood to only a small extent. The Carbamoylation of these amino acids might be due to contributions other than the haemoglobin, possibly Plasma Proteins and Enzyme Proteins or even erythrocyte membrane proteins as demonstrated by Krishna Sharma of DRDE, Gwalior. It was also observed that Valine was a major contributor as N-terminal Valine of those tissues, in case of kidney; both Valine & Alanine were equally Carbamoylated.

The number of Carbamoylated amino acids showed a drastic change in the Autopsy Samples within a month after exposure. From the Table 7.4 it may be observed that out of 7 amino acid carbamoylated, only 3 amino acid such as Valine, Glycine & Alanine showed their presence in the tissues of 1985. None of the samples after 1984 showed carbamoylation of Threonine, Hydroxyproline, Methionine or Phenylalanine. No tissue sample after 4th December 1984 showed Carbamoylation of Threonine (Table 7.5). It also may be observed that Valine showed its presence in 57 samples, Alanine in 16, Glycine in 11, Methionine, Phenylalanine 3 each and Threonine, Hydroxyproline 2 each during 1984 (Table 7.3). After the first quarter of 198, none of the samples showed N-carbamoylation of any amino acid, except valine (Table 7.4 & 7.6) in blood. Summary of Clinical & Autopsy Findings N-Carbamoylation and Tank Residue Compounds are shown in Table 7.7.

Quantitative Estimation of Blood MVH

Since valine carbamoylation was observed significantly, quantification studies were carried out. On DB-5 capillary column a linear response was observed with Ion Trap Detector between 10 to 300 ng of methyl valine hydantoin (VMH) according to peak area of m/z 114 and 157 fragment ions with a minimum detection limit of 9.3 ng. Whereas, this detection limit was much lower in case of HPLC method where it was recorded 20 ng, the VMH peak eluting at 15.64 min on C-18 column gave a low response with ultraviolet detector at 210 nm. However, the sensitivity of GC-NPD was observed much higher with a detection limit of 1 ng with a linear response upto 32.91 ng. Many of the samples were first studied on GC-NPD due to non installation of GC-ITD. On EGSP-Z column VMH was eluting at 3.14 min and the resultant peak was uniform and symmetrical with no tailing. During quantitation response factor was derived for every set of analysis by taking one or two known concentration allowing a tolerance limit of 10 percent. Some samples of December 1984 collection ere also analysed. Solvent blank and control sample were also analysed to check the detector response.

The results obtained on GC-NPD were verified with GC-ITD, and a good agreement was obtained between the two methods in quantitation. The data presented only GC-ITD results. This GC-ITD technique was adhered due to its greater specificity.

There was much variation in the concentration of carbamoylated valine between December 1984 samples and September 1985 samples. In December 1984 a mean concentration 1.64 mg/ml was observed against 0.18 mg/ml of 1985 (Table 7.8).

Table 7.8. MVH concentration in 1984 and 1985 autopsy blood samples (µg/ml)

Date	Case	Positive MVH	Percent (%)	Mean ± SD
3-12-84	33	19	57	1.0352 ± 0.8137
4-12-84	16	11	48	3.1249 ± 4.7535
5-12-84 to 31-12-84	12	5	61	0.6909 ± 0.6381
December 1984	**61**	**35**	**57**	**1.6428 ± 2.8451**
1-1-85 to 31-3-85	6	3	50	0.1841 ± 0.1506
1-4-85 to 30-6-85	9	3	33	0.0689 ± 0.0519
1-7-85 to 30-9-85	9	3	33	0.2954 ± 0.4202
1-10-85 to 31-12-85	4	-	-	-
Total 1985	**28**	**9**	**32**	**0.1828 ± 0.2452**
Total 1984 & 1985	**89**	**44**	**49**	**1.3442 ± 2.6012**

The twenty blood samples which showed positive for carbamoylation out of 35 blood samples of 1987 collection gave a mean value of 1.0352 µg/ml, whereas the average of 4th December samples was higher accounted 3.1249 µg/ml. The 5 positive samples collected between 5th - 31st December gave a mean value of 0.6909 mg/ml (Table 7.8), from the same table it may also be observed that there was no consistency between the concentration and collection period. The first quarter of 1985 samples showed a mean value of 0.1841 mg/ml, second quarter 0.0689 mg/ml and the third quarter 0.2954 mg/ml. There was no significant difference between the sex factor and the concentration of carbamoylated valine (Table 7.9). This observation was consistent with that of number of samples carbamoylated in female group, indicating faster detoxification of carbamoylated amino acids.

Table 7.9. Sex wise distribution of MVH in autopsy samples (µg/ml)

Date	Male				Female			
	Cases	MVH	%	Mean ± SD	Cases	MVH	%	Mean ± SD
3-12-84	20	11	55	1.3666 ± 0.8390	13	8	61	0.5796 ± 0.5359
4-12-84	12	7	58	4.0059 ± 5.8015	4	4	100	1.5830 ± 1.7394
5-12-84 to 31-12-84	5	2	40	0.4107 ± 0.2831	7	3	42	0.8777 ± 0.8021
Dec 1984	37	20	54	2.1948 ± 3.5977	24	15	62	0.9068 ± 1.0372
1-1-85 to 31-3-85	4	2	50	0.1010 ± 0.0625	2	1	50	0.3505
1-4-85 to 30-6-85	6	2	33	0.0957 ± 0.3323	3	1	33	0.0154
1-7-85 to 30-9-85	3	2	66	0.4219 ± 0.5072	6	1	16	0.425
1-10-85 to 31-12-85	2	-	-	-	2	-	-	-
1985	**15**	**6**	**40**	**0.2062 ± 0.2835**	**13**	**3**	**23**	**0.1361 ± 0.1861**
1984-85	**52**	**26**	**50**	**1.7358 ± 3.2532**	**37**	**18**	**47**	**0.7783 ± 0.9866**

Interestingly, a significant variation was observed in the concentration of carbamoylated Valine to that of age factor. The children showed a concentration of 0.6188 mg/ml which was much lower than adolescent (3.9516 mg/ml), whereas, a middle concentration was observed in adult group with a mean value of 1.0444 mg/ml (Table 7.10).

It is noteworthy that in the first month out of the total of 61 samples, Carbamoylation of glycine and alanine residues was seen in 9 and 10 cases respectively and practically none thereafter (Table 7.3). The presence of N-

Table 7.10. Age wise distribution of MVH in 1984 and 1985 blood samples (µg/ml)

Date	Up to 5 years				More than 5 to less than 15 year				More than 15 years			
	Cases	Positive MICT	%	Mean ± SD	Cases	Positive MICT	%	Mean ± SD	Cases	Positive MICT	%	Mean ± SD
3.12.84	7	5	71	0.7893 ± 0.5197	7	4	57	0.8076 ± 1.2128	19	10	52	1.2429 ± 0.7787
4.12.84	3	2	66	0.8316 ± 0.9714	4	2	50	10.2397 ± 9.254	9	7	77	1.7473 ± 1.5454
5.12.84 to 31.12.84	3	1	33	0.1716	-	-	-	-	9	4	44	0.8207 ± 0.6562
December 1984	**13**	**8**	**61**	**0.7226**	**11**	**6**	**54**	**3.9516 ± 6.4603**	**37**	**21**	**56**	**1.3336 ± 1.0816**
1.1.85 to 31.3.85	4	2	50	0.2036 ± 0.2076	1	-	-	-	1	1	100	0.1452
1.4.85 to 30.6.85	4	-	-	-	1	-	-	-	4	3	75	0.0689 ± 0.0519
1.7.85 to 30.9.85	5	-	-	-	-	-	-	-	4	3	75	0.2954 ± 0.4202
1.10.85 to 31.12.85	-	-	-	-	-	-	-	-	4	-	-	-
Total 1985	**13**	**2**	**15**	**0.2036 ± 0.2076**	**2**	**-**	**-**	**-**	**13**	**5**	**53**	**0.1769 ± 0.2698**
1984-85	**26**	**10**	**28**	**0.6188 ± 0.5625**	**13**	**6**	**46**	**3.9516 ± 6.4603**	**50**	**56**	**56**	**1.0444 ± 1.0691**

Carbamoylation of glycine in the tri-peptide of glutathione raises the other possibility of adjacent cysteine, taking a major part in the process of **S-Carbamoylation**.

Amongst the viscera, 11/14 samples of lung were strongly positive for MVH and fewer in liver (4) and spleen (3). The relative absence of the other carbamoylated amino acids in the viscera suggests that perhaps even the visceral MVH could be due to "contained blood" in the heavily congested haemorrhagic lung.

It is noteworthy that in addition to carbamoylation of valine, 6 more amino acids showed evidence of carbamoylation. These were glycine, alanine, threonine, hydroxyproline and phenylalanine. Interestingly enough in as many as 12 samples of blood non-methylated valine hydantoin was detected. It is not clear whether this is a result of in-vivo de-methylation due to the action of methyl transferases or due to presence of HNCO extra-carporeally in the inhaled gases. If so, it confirms our hypothesis that there was thermal decomposition of MIC in the tank and as described by Blake and Ijadi-Maghsoodi (1982), HNCO could be formed. There is all the more reason to imagine that the subsequent stage of thermal decomposition to HCN could also have taken place. Being lighter its distribution may not be uniform, as in the case of Tank residue compounds or even MIC. This might have accounted for variable deaths. It is possible that initially some of the deaths could be due to HCN as well. This is supported by the increased cyanide levels of early autopsy blood samples undertaken in this study.

The carbamoylation of glycine amino acid is particularly noteworthy in December 1984 samples. As it is an end-terminal amino groups of tri-peptide glutathione (GSH) anchored in the erythrocyte membrane, one can imagine it to be the first to be attacked even before haemoglobin. The mopping up might have also involved cysteine residue of the tri-peptide of GSH between glutamic acid and glycine. As per the information gained from the article of Cohen and Oppenheimer (1977), S-Carbamoylation is a rapid and reversible reaction. The observation of acidosis in the victims could have also contributed to the greater S-Carbamoylation in the more acid pH range. Unfortunately, all our attempts since 1988 to demonstrate S- carbamoylation were unsuccessful. In the meanwhile, the pioneering observations on Bailie and Slatter (1991) gave for the first time support to our quest of S-Carbamoylation of blood. However, further work with GC-MS did not yield successful results. Therefore, for the present it is tentatively surmised that next to valine or glycine, perhaps the -SH groups of cysteine (on the side chain) of the glutathione might be responsible for the malfunctioning of -SH containing enzymes like aldolase, Rhodanese etc, leading to the specific clinical symptomatology (air hunger, muscle weakness, cramps, inability to work etc.) in the Bhopal victims and the dramatic response to sodium thiosulphate.

The discrepancy between TNBS technique of free amino groups demonstrated by Ramaiah and Roman Reddy on the one hand and the DRDE and Medicolegal Institute on the other, may perhaps be explainable on the basis of mopping of MIC by -SH groups of erythrocyte bound glutathione.

But it is unfortunate that none of our attempts to demonstrate the equally important S-Carbamoylation of the -SH residues of erythrocyte- bound glutathione etc were successful. The preparation of S-carbamoylated cysteine reference standard could not be achieved. In the course of some of the discussions with Mazumdar, then biochemist in the Institute of Pathology, New Delhi, it was realised that:

- "At present there is an immediate need to make standards of S-Carbamylated cysteine and show its presence in blood and tissues of gas affected individuals. The urgency for this has already been repeatedly stressed by Sriramachari."
- "Preparation of S-carbamoylated cysteine is quite complex and involves the protection of N-terminal groups of cysteine. A possible way to achieve this would be to use glutathione as the cysteine source. Glutathione (L-glutamyl-l- cysteinyl glycine) when reacted with excess MIC may carbamylate cysteine at the S-position and glycine at the N-position. This can then be hydrolysed and cyclysed to yield glycine hydantoin and S-carbamoylated cysteine."
- "S-Carbamoylation of cysteine, even if done after protecting the N-terminal group, does not form hydantoins by cyclisation. It can neither be detected by the existing GC-ITD (GC-MS) system. Even if S- carbamoylated cysteine is prepared in the laboratory, it is not volatile and cannot be detected, conversion of this S-carbamoylated cysteine to a highly volatile substance may be necessary for detection by GC-MS. Either the present GC-ITD system has to be upgraded to include Chemical Ionization Detection system or alternatively the spectra can be taken to a place where a more complete GC-MS is available."

Such information could have explained the discrepancy mentioned earlier between relatively higher TNBS reac-

tions and the lower yields of MVH. The significance of this lacuna becomes all the more apparent after the publication of Baillie and Slatter (1991), although there was sufficient indication in the literature, including the work of Gwalior group. It may however be pointed out that many of these studies are based on reactivity with DTNB (analogous to our TNBS data) and not the actual demonstration of S-carbamoylation of cysteinyl residue. S-carbamoylation being a relatively labile process and the ubiquity of the tri-peptide glutathione, the rapid distribution of MIC in the body might have been facilitated. It is not known with certainly whether the reversible component, of glutathione carbamoylation would show a preferential affinity for other -SH groups of proteins and enzymes, such as liver proteins, rhodanese, aldolase or choline-esterase. Such a mechanism would have explained the link-up between Carbamoylation of glutathione, muscle weakness due to inhibition of aldolase and accumulation of cyanide in the body due to inhibition of rhodanese.

Repeated attempts to draw upon the expertise and help of eminent biochemists of the country in the AIIMS, CCMB etc did not lead to further elucidation on the binding of Isocyanates and Sulphydryl groups as suggested by Torchinsky (1991). Our efforts to continue the project or embark on a new collaborative one with DRDE, Gwalior have not yet received support from the ICMR. It is feared that this last facet of our understanding of the biochemical lesion of the Bhopal Disaster may remain an untold story.

In Conclusion, this study of demonstration of binding of MIC to end terminal amino groups of Hb and tissue proteins is more than of academic interest. In the first place it has established that MIC successfully crossed the alveolar-capillary barrier, contrary to circulars of Union Carbide Corporation. More importantly the steps involved in the procedure have been systematically unraveled. Firstly, by the demonstration of reduction of end terminal amino acids of the blood and several tissue proteins. Secondly, it opened up new avenues for exploring the Pathophysiology of MIC caused ARDS by BGA analysis and elevated 2-3 DPG levels, compensatory rise of Hb levels. It is significant that these physiological disturbances are beyond the quantum of binding of MIC to Hb. The observations of eminent scientists like Dr. Bucher are noteworthy. This unique positive Indian finding was appreciated by Bucher in a personnel communication to Ramachandran of DRDE shown below:

> *"I do find this convincing evidence that reactive MIC could cross the alveolar blood barrier as extremely important. Although, the significance of small amounts of MIC gaining access to blood stream, cannot be judged in relation to the substantial respiratory tract injuries"*

Apparently there is a wide difference in the positivity of the results between the two methods followed for tracking of MIC in Bhopal Victims. The wide discrepancy between the relatively high percentage of 25-40% reduction of End-Terminal Free Amino Groups, referred to earlier and only 1-2 % N-Carbamoylation of the 'Valine Residues' is probably due to S-Carbamoylation of Glutathione and other biomolecules to a substantial extent. By contrast, MVH positivity in Autopsy Blood Samples, (as compared with the foregoing Clinical material), was not only much higher, but was associated with a couple of other end-terminal Amino Acids, probably related to affected Serum Proteins & Erythrocyte Membranes (vide Krishna Sharma of DRDE). A majority of +ve cases occurred during the initial few weeks of the 1st Quarter after the Disaster, although the respiratory symptoms continued upto period of 8-9 mounts. A few 'late autopsies' were MVH +ve, well beyond the life span of the original erythrocytes. Since N-Carbamoylation is an irreversible process, it is tempting to speculate that such examples of late MVH +vity 'probably represent recycling from more labile radicals of S-Carbamylated MIC groups and Glutathione molecules. These changes are also co-terminal with the distribution of the Tank Residue Product. In brief, these studies have been very useful and informative in explaining the physiological disturbances associated with ARDS in Bhopal Victims. It's an unparallel event in the history of chemical disaster.

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Levels of free Amino Groups in Hemoglobin of Victims of Bhopal Gas Tragedy

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Summary

The free amino group s in hemoglobin of' victims of Bhopal gas tragedy were estimated by the method of Robert Fields (1). 6 to 67% decrease, in the tree amino groups in hemoglobin of' those patients were observed .compared to unexposed controls. This decrease in free amino groups in hemoglobin of gas victims 'might be due ·to, carbamylation by methyl isocyanate or its demethylated derivative namely lsocy1anate.

Introduction

Methyl isocyanate was supposed to be the major gas that killed thousands of people of Bhopal and made many thousands invalid.

Hemoglobin is the major protein of the body which constant is exposed in the lungs to outside atmosphere. Its amount in the adult body with 5 liters of blood is about 850 gms assuming that it consists about 34% of RBC volume and RBC occupies 50% blood volume. The, duration of life of RBC is about 4 months i.e. DBC once made, lasts in the body' for about 4 months. There fore, it 1s fairly safe to assume that the major effects of methyl isogamete inhaled by' the people of' Bhopal may be related to its effects on hemoglobin.

Methyl isocyanate is highly reactive compound and one of 'the major reactions it can have with proteins are to Carbamoylation the free NH2 groups (2), Sulphydryl groups (3) carboxy1 groups (2) phenolic hydroxyl 1 groups (4), imidazole groups (5) and phosphate groups (6).

We, therefore, looked for the blockage of free amino groups in hemoglobin.

Materials and Methods

All chemicals that were used were of analytical grade. Trinitro benzene sulphuric acid (TNBS) was from Fluka AG, CH-9470, Buchs, packed in Switzerland.

Blood samples were collected from normal and victims of Bhopal gas tragedy (adult. blood and cord blood) and were stored at 0-4°C till they were processed.

All the procedures were done at 0-4°C except the estimation of free amino, groups in hemoglobin which was done at room temperature. Water that was used was always double distilled and was de-ionized.

Preparation of RBC Lysates

To 0.5 ml of blood in a centrifuge tube was added 1 ml of phosphate buffer saline (0.9% NaCl in 50 mm phosphate butter pH 7.2) (PBS) and was mixed gently It was then centrifuged at 2000 rpm for 10 min. The, supernatant was discarded and to the pellet of cells, 2 ml of PBS was added and was mixed gently. It was again centrifuged at 2000 rpm for 10 min. To the pellet of cells, double distilled and de-ionized water was added to a final volume up to 4.5 ml and was kept for 15 min. with occasional vigorous mixings. The lysate was centrifuged at 4000 rpm for 30 min. and the supernatant was collected which was taken as RBC lysate.

Dialysis of RBC Lysates

RBC lysates were dialyzed against double distilled and de-ionized water for about 12 hrs with a change in water

once in between. It is thus free of amino acids which react with TNBS. These dialyzed RBC lysates were the source of hemoglobin for the determination of free amino groups.

Estimation of Free Amino Groups of Dialysed RBC Lysates:

Hemoglobin in RBC lysate constitutes 99.2% of its total protein as calculated in Table 1. So the estimation of free amino groups of dialyzed RBC lysates by the rapid method with TNBS (1) was considered as the levels of free amino groups of hemoglobin. Estimation to free amino groups in the dialyzed RBC lysates by TNBS method was based on its reaction with amino groups to tom trinitrophenly 1 amino groups in alkaline medium at pH 9.5 (0.05 M $Na_2B_4O_7$ in 0.05 M NaOH). The reaction was stopped by adding 2 ml of 1.5 mM. Sulphite in 0.1 M NaH_2P0_4 to 1 ml reaction mixture", exactly after 5 min sulphite forms a complex with trinitrophenly 1 amino groups which absorbs at 420 nm. The concentration of TNBS during the reaction was 21.568 mM. In view of the fact that TNBS itself contributes some blank in alkaline medium at 420 nm and the fact that hemoglobin also absorbs at 420 nm. the reading at 420 nm after stopping the reaction exactly after 5 min with sulphite in acid medium followed by addition of the same- amount of hemoglobin as was used for the reaction was taken as proper blank. 1.5 to 3 n moles of hemoglobin was used for this reaction and in this range the reaction was linear.

Results And Discussion

16 samples of adult blood and 11 samples of cord blood of victims of Bhopal gas tragedy were analysed for free amino groups of hemoglobin for TNBS reaction and were compared with the values to tree amino groups of hemoglobin of normal unexposed controls. The results are shown in Table II and Table III. Normal values of free amino groups for adult blood hemoglobin and cord blood hemoglobin were found to be 8 and 10 moles of amino groups/mole of hemoglobin tetramer respectively (Molecular weight of hemoglobin tetramer was taken as 66,668).

These values were calculated using molar extinction coefficients of chromogen at 420 nm after DBS reaction at 22,000 (1) and of hemoglobin tetramer at 420 mm as 415.4536 x 103 which is found 7.751 times the, value of hemoglobin at 540 nm (12) based on the analysis of 5 samples of hemoglobin In all blood samples that were ana1ysed the decrease in free amino groups of Hemoglobin was observed. MIC might have been absorbed through lungs into blood, where it might have reacted with hemoglobin along with other proteins in the blood and might have formed carbamy1 derivatives and thus reduced the, free amino groups in hemoglobin since hemoglobin consists of 34% of RBC volume, the major affected protein in .the blood could be hemoglobin. the free amino groups in hemoglobin that could have been carbamylated are N-terminal valine and amino groups to lysine residues in hemoglobin this could have resu1ted 1n extensive detects of hemoglobin functions like important, of carriage of carbon dioxide by N-terminal valine (13) and oxygen delivery to the tissues (14).

Table 1. Protein and Water Content of Human Erythrocyte

Component	mg/ml	Reference
Water	721.0 ± 17.3	7 and 8
Hemoglobin	361.8	8 to 11
Insoluble stromal Protein	6.3	8
Protein from enzymes	2.9	8

% Hemoglobin present in RBC lysates calculated from the table:

$$\frac{361.8 \quad X \quad 100}{361.8 \quad + \quad 2.9} = 99.2\%$$

Table 2. Percent Decrease in Free Amino Groups of Adult RBC Lysates of affected People Compared to Normals.

Normal value = 8 moles of free amino groups/mole of hemoglobin tetramer.

Sample No.	% decrease in NH_2 group's
1	25.9
2	67.1
3	41.3
4	24.1
5	9.4
6	30.3
7	19.7
18	13.4
19	34.9
118	58.5
R – 2064	5.8
R - 2066	13.0
R - 2081	11.6
R - 2069	31.5
R - 2070	19.4
R - STH	25.9

Table 3. Percent Decrease in Free Amino Groups of Cord RBC Lysates of affected People Compared to Normal.

Normal value = 10 moles of free amino groups / mole of Hb tetramer.

Sample No.	% decrease in NH_2 group's
1	36.3
2	66.0
3	64.7
4	61.0
5	46.9
10	62.8
11	44.2
12	9.7
14	31.8
16	8.2
16	51.2

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Journal of Chromatography, 426 (1988) 239–247
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4080

GAS CHROMATOGRAPHIC STUDIES OF THE CARBAMYLATION OF HAEMOGLOBIN BY METHYL ISOCYANATE IN RATS AND RABBITS

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(First received October 16th, 1987; revised manuscript received December 1st, 1987)

SUMMARY

Carbamylation of the N-terminal valine of haemoglobin with methyl isocyanate in rats and rabbits has been demonstrated in vitro and in vivo by gas chromatography. N-Methylcarbamylated haemoglobin, converted by cyclization into 3-methyl-5-isopropylhydantoin, has been quantified by gas chromatography. Standard hydantoin was synthesized, chemically characterized and used for calibration. The method is simple and reliable in the concentration range 0.06–2 nmol. Carbamylation of haemoglobin by methyl isocyanate in vivo in rats can be identified only above a dose of 1.05 mg/l in inhalation exposures. It is inferred that methyl isocyanate in the "active" form crosses the alveolar and erythrocyte membranes and carbamylates the haemoglobin.

INTRODUCTION

One of the hypotheses put forward for sudden deaths due to methyl isocyanate (MIC) exposure is tissue anoxia due to carbamylation of haemoglobin. Our attempts to substantiate this hypothesis by electrophoresis and isoelectric focusing of MIC-exposed haemoglobin did not give any positive conclusion and we therefore chose gas chromatography (GC) as a possible technique for elucidation of the hypothesis.

It has been shown by Manning et al. [1] that cyanates react with the N-terminal valine of haemoglobin, which can be converted into 5-isopropylhydantoin or valinehydantoin and determined by GC. The method was used for monitoring

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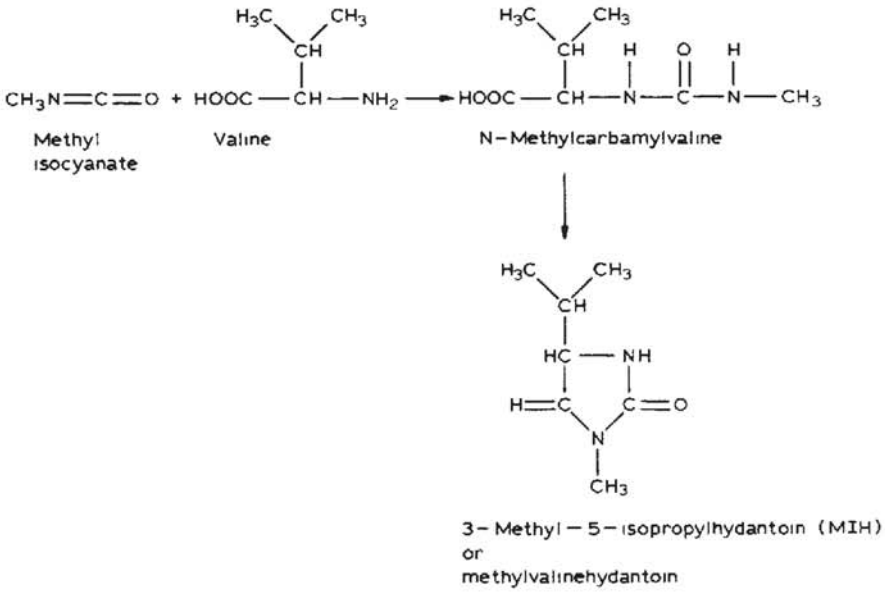

Fig 1. Reaction mechanism of haemoglobin carbamylation.

carbamylation levels in sickle cell anaemic patients subjected to cyanate therapy. Lee [2] extended the study to the anti-sickling properties of MIC, which he reported to be much faster. Lee clearly demonstrated that MIC reacts irreversibly with the α-amino group of the N-terminal valine in haemoglobin to the exclusion of sulphydryl and ϵ-amino groups. The above studies were carried out in vitro by the addition of cyanate or MIC to human blood. The in vivo carbamylation by cyanate has been reported by Crist et al. [3] and Papayannopoulou et al. [4], but no attempts at the in vivo determination of carbamylated valine with MIC have previously been reported.

We report here both in vivo and in vitro studies of haemoglobin carbamylation with MIC in rats and rabbits. The reaction sequences considered in this study are depicted in Fig. 1.

EXPERIMENTAL

Materials

All the reagents used were of analytical-reagent grade and L-valine was obtained from Sigma (St. Louis, MO, U.S.A.).

Synthesis of MIC

Acetyl chloride and sodium azide were reacted in benzene in the presence of a phase-transfer catalyst as described previously [5]. The purity of the product (b.p. 38.0°C) as checked by GC was 99.5%.

Preparation of standard 3-methyl-5-isopropylhydantoin (MIH)

L-Valine (468 mg; 4 mmol) was dissolved in 20 ml of distilled water and the pH adjusted to 8.0 with 0.1 *M* sodium peroxide. Chilled MIC (228 mg; 4 mmol) was added and allowed to react for 2 h with stirring at room temperature. Glacial

acetic acid (7 ml) and concentrated hydrochloric acid (13 ml) were then added and the solution was heated to 100°c for 1 h. The solvent was removed under reduced pressure and the residue dried overnight over sodium hydroxide in a vacuum desiccator. The melting point of the product was 210°C. The identity of MIH was established by mass spectrometry (MS) [$M^+ = m/z$ 156, $(M-42)^+ = m/z$ 114]. The purity of the compound as checked by GC was 99.5%.

MIH was also prepared by methylation of 5-isopropylhydantoin by the method of Carington and Waring [6]. 5-Isopropylhydantoin for this purpose was prepared as reported [1] by carbamylation of L-valine with potassium cyanate.

Exposure chambers

An all-glass static exposure chamber of volume 21.5 l was specially designed and has been described in detail elsewhere [7]. Required concentrations of MIC were obtained by introducing known amounts of MIC into the chamber through a detachable side-arm. A circulating pump enabled a uniform concentration to be attained. In a separate experiment, the concentration of MIC was monitored by GC and found to be constant over a period of 30 min. The exposed doses were calculated in terms of LC_{50} for 30 min for Wistar rats of weight 200 ± 20 g. Rabbits were exposed in an all-stainless-steel static exposure chamber of volume 35 l. The exposure dose was 3 mg/l for 30 min. New Zealand–Dutch cross rabbits of weight 1000 ± 50 g were exposed one at a time.

Blood was collected from rats and rabits 1 h prior to and 30 min after the exposure in heparinized centrifuge tubes. The haemolysate was prepared as described [8]. The haemoglobin concentration was measured spectrophotometrically [9].

Processing of haemolysate from in vivo experiments

To 1.0 ml of haemolysate prepared from exposed and control animals were added 5 ml of 2% hydrochloric acid in acetone at 4°C and mixture was vortexed thoroughly. The tubes were centrifuged at 2000 *g* for 3 min and the supernatant liquid was discarded. The whole globin pellet was resuspended, washed four times with cold acetone as above and finally with 5 ml of cold diethyl ether, then centrifuged again. The precipitate was dried by placing the tubes in a beaker of warm water at 45°C.

The carbamylated product was dissolved in a mixture of 0.5 ml of 50% glacial acetic acid and 0.5 ml of concentrated hydrochloric acid and kept at 100°C for 1 h, cooled immediately, neutralised by adding 0.8 ml of 10 *M* sodium hydroxide solution and 0.5 ml saturated sodium chloride solution and the contents were mixed. The pH of the solution was maintained between 3 and 5. Then 5 ml of ethyl acetate were added to the hydrolysate and MIH was extracted by mixing the contents of the tube for 1 min on a vortex mixer. After separation of the phases by centrifugation, 4 ml of the ethyl acetate phase were placed in a heavy-walled Pyrex tube. Ethyl acetate was removed by evaporation to dryness and the tube was placed overnight in an evacuated desiccator over sodium hydroxide for removal of traces of water. The dried residue was dissolved in a known volume of

ethyl acetate and 1 μl of the solution was injected on to the GC column. A similar procedure was followed for all other samples.

Processing of haemolysate from in vitro experiments

The haemoglobin concentration was adjusted to 160 mg/ml of normal rabbit haemolysate. In every instance 2.5 μmol of haemoglobin were taken and various amounts of MIC, as given in Table II, were added. The reaction was allowed to proceed for 2 h at room temperature with constant stirring. The haemolysates were then processed for extraction of the carbamylated product and cyclization to hydantoin as described above. Similarly, in vitro MIC-treated samples of rat haemolysates were processed to study the carbamylation. In vitro studies with excess of MIC (two-fold) were also carried out.

GC analysis

A Perkin-Elmer Model 3920-B instrument was used with flame-ionization detection (FID). A stainless-steel column (180 cm × 2 mm I.D.) packed with 15% OV-225 (Analabs, North Haven, CT, U.S.A.) coated on 100–120 mesh Chromosorb W HP was employed. The injection port and detector block were maintained at 230 and 225°C, respectively, and the column oven temperature was 220°C. Nitrogen was used as the carrier gas (at a flow-rate of 30 ml/min). Air for FID was supplied at 300 ml/min and hydrogen at 30 ml/min. In all analyses, 1-μl samples were injected and peaks recorded on Shimadzu Chromatopac C-R3A data processor. The FID amplifier attenuation was 8 × 10 and the C-R3A × 1 or × 2.

Calibration graph for standard MIH

A stock solution of standard MIC was prepared at a concentration of 1 mg/ml. Solutions of various concentrations in the range of 0.06–2.0 nmol/μl were prepared by taking known volumes of stock solution and diluting it with freshly dried ethyl acetate. A 1-μl volume of each was then injected on to the GC column and the MIH peak height was recorded. Average peak heights from replicates were plotted against concentration of MIH.

Calculations

In every instance the concentration of MIH in μmol/ml of blood/haemolysate (C) was calculated using the observed peak height, the corresponding MIH concentration and the dilution factor. The degree of carbamylation was expressed in terms of the percentage of total haemoglobin utilized for carbamylation using the equation:

$$\text{percentage carbamylation} = \frac{C\ (\mu\text{mol})/4}{\mu\text{mol haemoglobin/ml blood}} \cdot 100$$

RESULTS AND DISCUSSION

The chromatogram of standard MIH is shown in Fig. 2. The retention time for MIH was 2.65 ± 0.15 min. The peak was narrow and symmetrical. The FID response was linear in the range of concentrations used for constructing the calibration graph. The peak-height reproducibility was within ±2 mm. Hence the determinations were considered to be satisfactory. The recovery of MIH in the ethyl acetate layer was found to be better than 92%. The losses in the extraction procedure were not taken into account in the calculations. The detection limit of MIH was 5 ng (0.03 nmol) at a signal-to-noise ratio of 8.

Typical chromatograms obtained for blood samples processed from control and exposed rats are shown in Fig. 3a and b and for rabbits in Fig. 4a and b. Figs. 3 and 4 clearly indicate that no other product interferes with the MIH peak. Data from in vivo experiments given in Table I for the exposed animals indicate that all the exposed animals showed a positive test for carbamylation, as confirmed by the presence of MIH. The detection limit of MIH was 1 LC_{50} exposure dose for rats. For lower than 1 LC_{50} doses, no carbamylation was detectable. As MIC is a sensory irritant, the rate of breathing of the animal would be reduced by reflux inhibition. Further, as MIC is a highly reactive moiety, interacting with all nucleophiles, the amount of MIC reacting with haemoglobin would be only a small fraction of the total MIC entering the biological system. The data in Table I for percentage carbamylation show less than 2% in all doses studied from 1 to 3 LC_{50}.

Fig. 2. Chromatograms of (a) solvent (ethyl acetate) and (b) 2 nmol of standard MIH. Retention time, 2.65 min; column, stainless-steel tubing (180 cm × 2 mm I.D.) packed with 15% OV-225 coated on 100–120 mesh Chromosorb W HP; temperature, 220°C. Other conditions as described in the text.

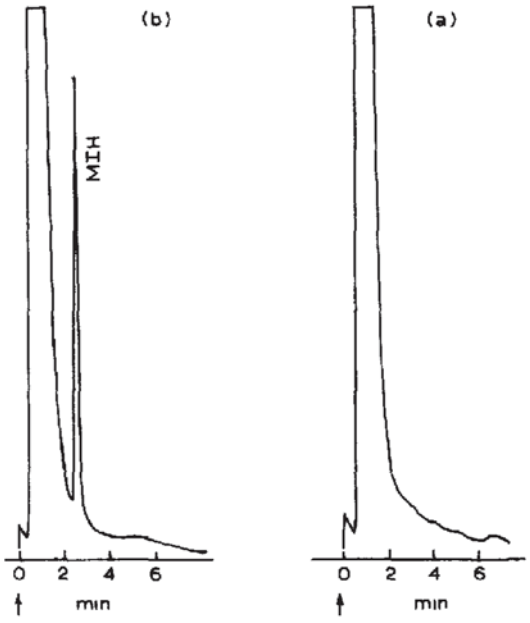

Fig. 3. Chromatograms of processed rat blood, (a) collected before exposing the rat to MIC and (b) collected after exposing the rat to a 3 LC_{50} dose of MIC for 30 min. In both instances 1 ml of blood was taken for processing and the final residue was diluted to 100 μl with ethyl acetate. Experimental conditions as in Fig. 2.

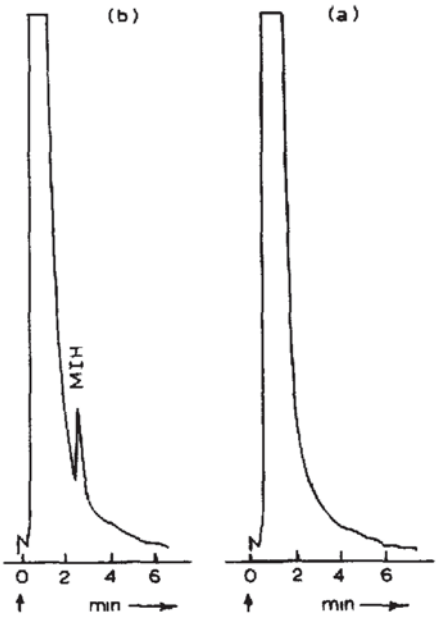

Fig. 4. Chromatograms of processed rabbit blood, (a) collected before exposing the rabbit to MIC and (b) after exposing it to MIC at a 3 mg/l inhalation dose. In both instances 1 ml of blood was taken for processing and the final residue was diluted with 300 μl ethyl of acetate. Experimental conditions as in Fig. 2.

For rabbits, the degree of carbamylation after exposure to an arbitrary dose of 3 mg/l is only 1%, confirming the above facts.

In vitro experiments were also carried out to study the dose dependence of the degree of carbamylation using rabbit haemolysates. The data given in Table II on the degree of carbamylation clearly indicate that the extent of carbamylation is high in vitro as compared to in vivo exposures.

The degree of carbamylation seems to be dose-dependent from the data given in Tables I and II. However, in the in vivo experiments it is not exactly proportional to the exposure dose. This is as expected, because MIC would enter the bloodstream from the lungs after reacting with the lung proteins, which reaction will be dependent on the residence time of MIC in the lungs; in the bloodstream also it is difficult to predict the amount reacting with haemoglobin and other proteins.

Work in this laboratory [10] with ^{14}C-labelled MIC administered by inhalation to rats showed that radioactivity was widely distributed in the liver, kidney and brain. This was also identified as the protein-bound radioactivity, thereby establishing the passage across the blood–tissue interface by MIC in the "active" form. A better correlation and enhanced carbamylation observed in the in vitro experiments further confirm the above.

The electrophoretic techniques carried out in our laboratory [11] and reported by Troup et al. [12] failed to help in identifying the carbamylation of red blood cells with MIC; the GC technique reported here could effectively establish carbamylatin in vivo. The sensitivity of the technique can be improved further by using a capillary column coupled with a nitrogen-selective detector. The method can then be successfully used for monitoring the lower degrees of carbamylation that may occur as a result of lower exposures in a chemical plant.

TABLE I

IN VIVO STUDIES ON CARBAMYLATION OF HAEMOGLOBIN IN RATS AND RABBITS AT VARIOUS EXPOSURE DOSES OF MIC

Exposed species	MIC exposure dose	Number of animals exposed	Number of animals showing carbamylation (detected by GC)	Average MIH formation in 1 ml of blood (determined by GC) (mean ± S.D.*) (nmol)	Degree of carbamylation (% of haemoglobin carbamylated)
Wistar rats	1 LC_{50}**	4	4	20.44 ± 1.96	0.2
	2 LC_{50}	5	5	80.09 ± 2.94	0.8
	3 LC_{50}	4	4	151.59 ± 3.40	1 5
Rabbits (New Zealand-Dutch cross)	3 mg/l	2	2	102.50 ± 2.24	1.0

*Losses in MIH extraction were <8% and neglected in the calculations. Average values for each animal for every exposure were obtained from replicate analyses and were used to find average MIH formation for a given dose for a given animal, as shown in this column.

**1 LC_{50} = 1.05 mg/l.

TABLE II

IN VITRO STUDIES ON CARBAMYLATION OF HAEMOGLOBIN IN RABBIT HAEMOLYSATE SHOWING THE DEPENDENCE OF THE DEGREE OF CARBAMYLATION ON THE AMOUNT OF MIC ADDED TO BLOOD LYSATE

In all instances 1 ml of haemolysate was processed.

Haemoglobin concentration in haemolysate taken for carbamylation* (10^3 nmol/ml)	Concentration of MIC added to lysate (10^3 nmol/ml)	Concentration of MIH in injected solution** (mean ± S.D.) (nmol/μl)	MIH determined by GC per ml of haemolysate*** (10^3 nmol)	Degree of carbamylation (% of haemoglobin carbamylated)
2.5	11.8	3.48 ± 0.15	6.9	69.0
2.5	5.8	1.54 ± 0.32	3.1	31.6
2.5	3.9	1.09 ± 0.20	2.2	22.5

*1 mol of haemoglobin = 4 mol of valine.
**The final residue was diluted with 2 ml of ethyl acetate and 1 μl of the solution was injected on to the GC column.
***Losses in MIH extraction were < 8% and neglected in the calculations.

CONCLUSIONS

Different workers had expressed and reiterated their belief that MIC would be completely hydrolysed to dimethylurea by the lung fluids and hardly any "active" MIC can enter the bloodstream [13,14]. Studies with radiolabelled MIC in this laboratory [10], and those of Hill et al. [15] using ^{14}C-labelled toluene diisocyanate, have confirmed the interaction of alkyl and aryl isocyanates with blood proteins. Our work clearly shows that MIC in the "active" form crosses the lung–blood barrier and reacts with haemoglobin in the circulating blood by carbamylating it.The degree of carbamylation by MIC is less than 2% in rats and rabbits by the inhalation route. Hence, at least one of the toxic symptoms after exposure to MIC, namely tissue anoxia, because of increased oxygen affinity of the carbamylated haemoglobin, is a possibility. The sensitivity of the technique can be improved by using a capillary column coupled with a nitrogen-selective detector.

ACKNOWLEDGEMENTS

The keen interest and encouragement of Brig. K.M. Rao, Additional Director, Defence R&D Establishment, Gwalior, India, during this study are gratefully acknowledged. Acknowledgements are due to Mr. R. Raghunandanan and Mr. A. Daniel for typing the manuscript.

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Report on the Analysis of Blood Samples of Bhopal Gas Victims for the Detection of Carbamylated Haemoglobin by Gas Chromatography

By

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Acknowledgement: *Authors of this Report wish to thank Brig K.M. Rao, Director, DRDE, Gwalior for his valuable suggestions during this work.*

Introduction

One of the hypotheses put forward for sudden deaths due to methyl isocyanate (MIC) exposure is tissue anoxia due to carbamylation of haemoglobin. It has been shown by Manning et al. (1) that cyanates react with the N-terminal valine of haemoglobin, which can be converted into 5-isopropylhydantoin or valine hydantoin and determined by gas chromatography (GC).The method was used for monitoring carbamylation levels in sickle cell anemic patients subjected to cyanate therapy. Lee (2) extended the study to the anti-sickling properties of MIC, which he reported to be much faster. Lee clearly demonstrated that MIC reacts irreversibly with the amino group of the N-terminal valine in haemoglobin to the exclusion of Sulphydryl and E- -amino groups. The above studies were carried out in vitro by the addition of cyanate or MIC to human blood. The in vivo carbamylation by cyanate has been reported by Crist et al. (3) and Papayannopoulou et al. (4), but no attempts were made at the in vivo demonstration of carbamylated valine with MIC earlier to our work (5). We have reported in vivo and in vitro studies of haemoglobin carbamylation with MIC in rats and rabbits. The reaction sequences in this study are depicted in Fig.1. Our work clearly showed that MIC in the 'active' form crosses the lung-blood barrier and reacts with haemoglobin in the circulating blood and carbamylate it. Hence, it was concluded that at least one of the toxic symptoms after exposure to MIC, namely tissue anoxia, because of increased oxygen affinity of the carbamylated haemoglobin, was possibility. GC technique was found to be sensitive to asure the degree of carbaylation upto 0.2%.

Samples

All the 160 human blood samples were provided by Dr. S. Sriramachari ICMR. New Delhi. Table 1 shows the Reference numbers and dates of collection of positive 20 out of the 160 samples.

Processing of the blood samples

All the samples were processed as per the method described by J.M.Manning et al. (l).

In every case 0.5 ml blood was taken. Haemoglobin was precipitated by adding 2% HCl in acetone. Precipitated haemoglobin was centrifuged, washed with cold acetone and diethyl ether and dried over warm water.

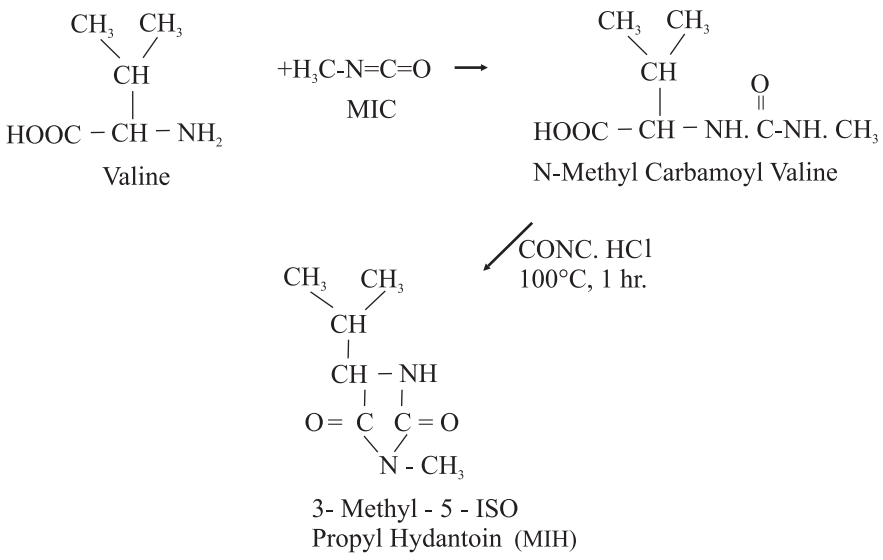

Figure 1. Reaction Sequence for MIH formation

Table 1. Details of the blood samples analysed by GC to check the carbamylation of haemoglobin due to MIC

Sample No.	ICMR Ref / Name	Carbamylation test by GC
2	10-1/10-2-85	+++
7	29/6-3-85	+
10	10A/2424-3	+++
15	S TH/5- 3-'85	+++
16	11-1/3-85	+
19	4-1/5-85	+
23	SS/7-5-85	++
25	4058/150/18-8-85	+
31	6A	+
33	7A	+
40	(4)	++
43	M.L.I.F/14-5-85	++
44	P.M.NO.27-85/12-1-85	+++
47	YDA /4063/158/C	+
50	4067/160	+
54	JS/P2/7-5-85/C	+
56	NLM	+
81	35	+
118	9	++
156	JDl18/-DO-/C	+

Dry precipitate was then disolved in 50% glacial acetic o acid and equal volume of cone. HCI and kept at 100 c for 1 hr. Then cooled immediately; neutralised by adding 10N NaOH and saturated NaC1 keeping the pH between 3 to 5.Ethyl acetate was then added to the hydrolysate and methyl valine hydantoin (MIH)formed was extracted by mixing contents of the tube for 1 min on vortex mixer. (MIH if formed .was extracted into ethyl acetate layer.)The solvent was then evaporated to dryness in vacuum desiccator over NaOH for 2 hrs. The dried residue was dissolved in ethyl acetate and the portion of this solution was injected into GC.

GC Analysis

In every case l/µ1 of the appropriately diluted solution of the extracted residue in ethyl acetate was injected onto 2 GC column. The analysis was carried out on 15 % OV-225 packed column of SS tubing (6 ft long and 2 mm i.d.)at 220°C. installed on Perkin Elmer model 3920-B.Flame ionisation detector(FID) with hydrogen flow at 30 ml/min and air flow at 300 ml/min was used for detection of the column efluents. The carrier gas was nitrogen at 30 ml/min flow rate. The injection o port and detector block temperatures were kept at 240°C. The peaks were recorded on Shidzu Printer-Plotter model C-R3A.

The peak for MIH was identified by comparing the retention time for standard MIH prepared by reacting L-valine with MIC and derivatising carbamylated product as described above. The retention time for standard MIH was 2.65 ± 0.15 min. Relative retention time for MIH w.r.t. valine hydantoin was 0.5.The mass spectrum for standard MIH was as shown in Fig.2.

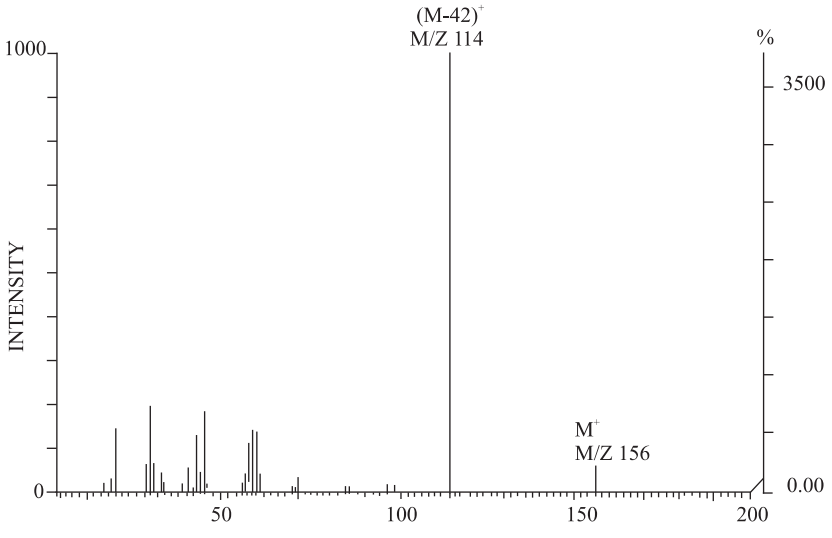

Figure 2. Mass Spectra of MIH (Standard)

Results

All 160 blood samples were processed under identical conditions and analysed on GC to check the presence of MIH Peak. The GC test results are given in Table 1 against each sample reference in terms of presence(+) or absence (-) of MIH peak as indicator of carbamylation of blood protein due to MIC inhalation. The chromatograms of solvent (ethyl acetate) and that of standard MIH are shown in Fig.3. The chromatograms of some samples with positive test are shown in Fig.4 and those with negative test in Fig.5. The chromatograms of control blood and control blood spiked with MIH are shown in Fig.6 and 7 respectively.In all 20 samples out of 160 showed clear presence of MIH.Sample no. 44.15.10 and 2 gave highly significant peak for MIH (indicated by +++ grading. Sample no.43, 40, 23 and 118 showed significant peak for MIH (indicated by ++ grading). While small amount of MIH is present in sample no.7,16,19,25,31,33,47,50,54,56,81 and 156. (indicated by + grading). Since the peak for MIH is quite sharp and symmetrical in nature the peak height can be considered to be proportional to the concentration and hence variation in peak height can be considered as an indicator of the degree of carbamylation of blood protein due to MIC.

The GC technique is simple and reliable in the concentration range of 0.06-2.0 nmol. The peak height reproducibility for MIH peak was with in the accuracy of ± 2 mm and the detection limit was 5 ng (0.03 nmol) at a signal to noise ratio of B.The recovery of MIH in the ethyl acetate layer was found to be better than 92%.

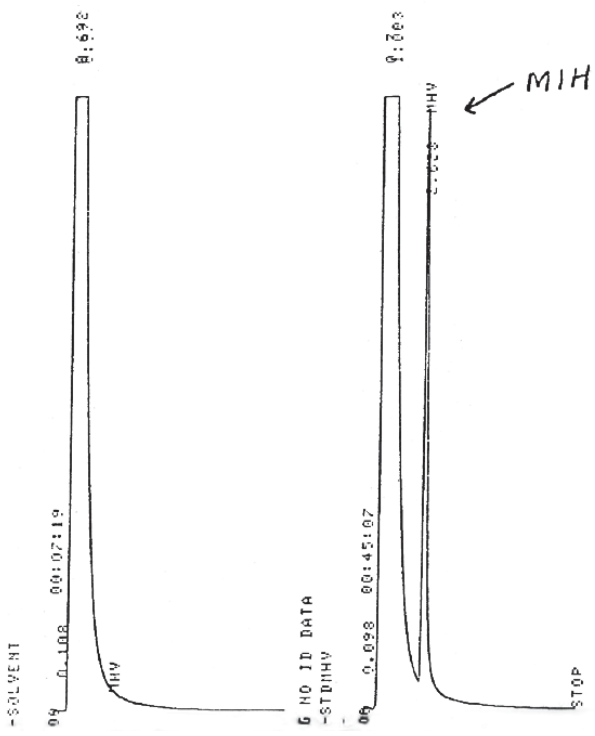

Figure 3.

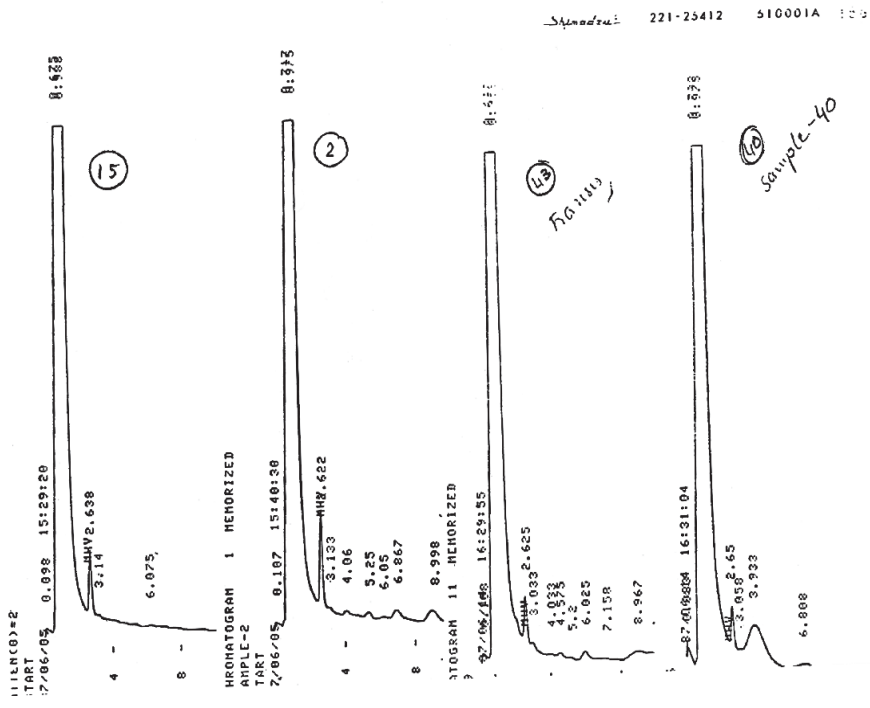

Figure 4.

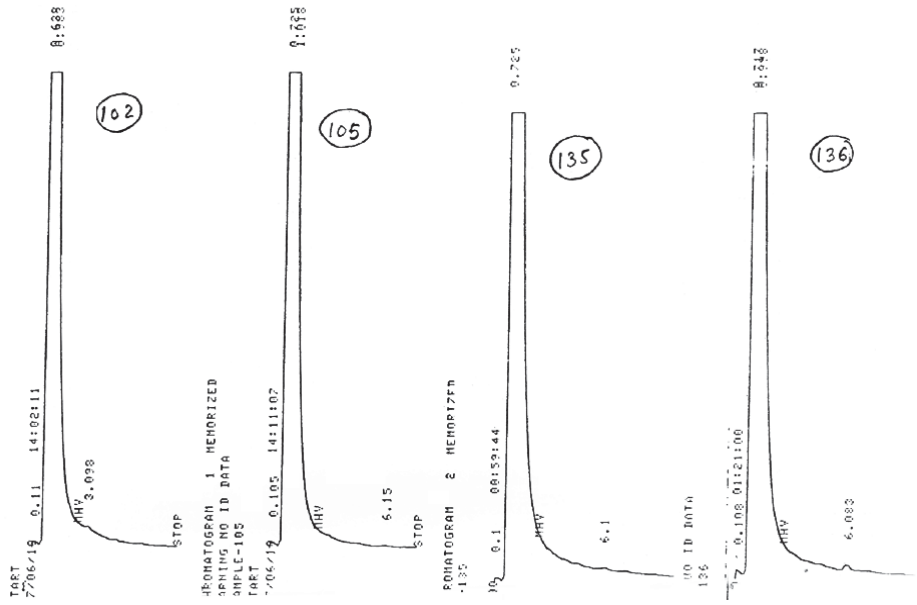

Figure 5.

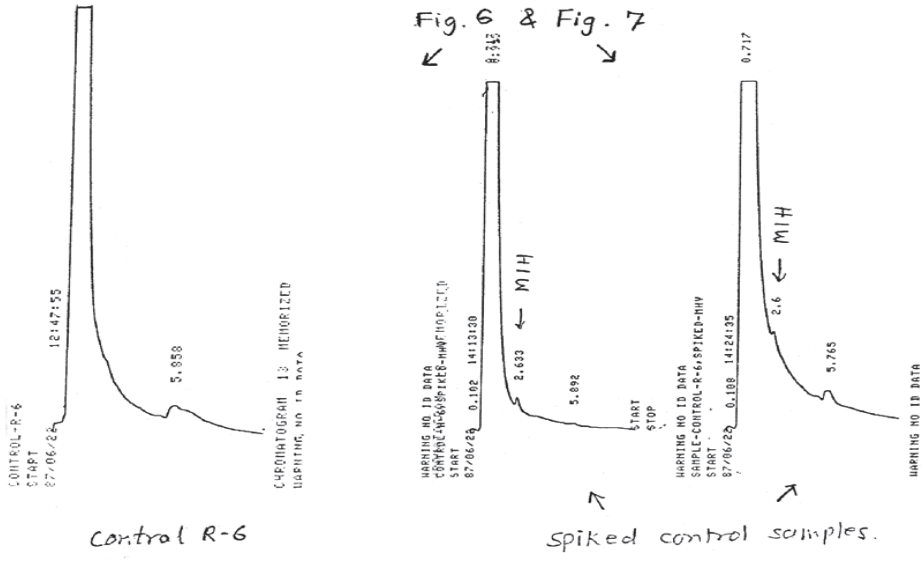

Figure 6 and 7.

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❑

CHAPTER

8 TANK RESIDUE ANALYSIS

As shown in the preceding Chapters, apart from **N-Carbamylation** due to binding of MIC to Hb and tissue proteins, sophisticated techniques like GC-MS revealed additional evidence of the MIC-derived compound/s, especially in some of the early autopsies. The details are already presented in Tables 7.3-7.5 in Chapter 7. Based on some of the initial observations, greater attention was given to the exact nature of the compounds and possible long-term hazard of ***'Recurrent Cyanogenesis'***. Firstly with progressive access to more refined equipments such as GC, GC-MS with ITD system, the composition of each one of the Tank Residue Constituents (TRCs) and their possible '***Nitrile-like Effects***' were investigated. Secondly, the entire range of 'extraneous chemicals' lurking in the bodies of Bhopal victims were correlated. At the outset, it must be stated that the TRCs also were present only in the first few weeks, as in the case of N-Carbamylation of the Amino Groups (Table 7.6).

I. Tank Residue Analysis

Physico-Chemical Properties of TRCs: All the three layers of TRC viz., top, middle and bottom layers were recorded for colour, odour and appearance. These were kept in 500 g capacity bottles. On opening the bottles a strong fishy amine like odour was present and all the three layers had brown colour in appearance. The layer 1 (bottom) was more solid and crystalline in nature than the other two layers of light brown colour, whereas layer 3 (top) was paste-like semi-solid and dark brown in colour. This indicates a definite variation in the chemical composition of all the three layers. The TRCs were analysed by TLC technique, using 200 x 100, 200 x 50 and 75 x 25 mm glass plates coated (0.25 mm) with silica gel-G (BDH Chemicals) and activated at 120°C. Chloroform, ethyl acetate, toluene and benzene as developing solvents in differential mixtures were used. After the chromatographic run the qualitative picture was recorded by using UV light (366 nm). The TLC run indicated the presence of about 11 compounds under UV light.

Following the TLC analysis, TRC samples were analysed on Sigma 300 GC-FID. For the analysis of amines, 8.9% TEPA, 1.1% KOH on porapak Q 80/100 mesh (2m x 1/8"OD) packed column was used. The 'head space' of the TRC was tested on Super-Q 80/100 mesh (Teflon 12' x 1/8"OD) packed column for the presence of cyanide. Nitrogen was used as a carrier gas at flow rate of 45 ml/min. Column, Detector and Injector temperature was 125°C, 230°C and 210°C respectively. Pressure lock syringe (Dyanatech Precision Sampling Corporation, U.S.A.) was used for drawing head space of the TRC vials capped with Teflon tapes. The rest of the compounds of TRC mixture were studied on 5% OV-17 on chromosorb W (HP) 80/100 mesh [glass lined tube (GLT) 6' x 1/8" OD]. Nitrogen 40 ml/min as carrier gas Isothermal column 170°C, injector 250°C and detector 300°C. On TEPA column, TRC showed presence of 1-2% MMA (RT 1.857 min), 1-2% DMA (RT 2.217 min) and 2-3% TMA (RT 5.50 min).

The OV-17 on GLT column showed presence of 12 compounds in all the three layers. The MICT was eluting at RT 4.225 min with normalised peak area percent of 47.05. The second major compound on normalised peak area which accounted 23.35 percent was DMI eluting at 5.217 min. The third significant compound was 2, 4-dione, accounted 15.18% with an elution time of 6.15 min. A total of 85% was occupied by these compounds. The TMU and DMU were eluting at 1.192 min and 1.658 min, but TMU was present in a higher ratio (6.23%) than DMU (1.39%). The amines came out as a clump and emerged with solvent.

Later studies on the TRC analysis were extended using GC-ITD for confirmation of the earlier analysis. GC-MS studies were carried out on a Varian model 3400 capillary GC-ITD model 800 of Finnigan MAT Ltd., UK. Column DB-5, 0.25 mm x 30 m, 0.25 ìm film thickness (J&W Scientific), helium (purity 99.95%) linear velocity 16 cm/sec and the column head pressure was maintained at 10 psi, oven 180°C to 230°C at 5°C/min (180°C for 1 min, and 230°C for 10 min), injector 250°C, transfer line 250°C ITD Scan mode m/z (mass to charge ratio) range from 40 to 600 amu (1 scan/ sec), and multiplier

voltage gain 105+ 150 volts were used for the study. The ITD was operated in an electron impact mode at 70 eVv with an ion source temperature at 216-220°C. The data system for the ITD was an IBM PC-XT with standard GC-MS software of Finnigan MAT for tuning, total ion chromatogram generation and library search routines.

The chloroform fractions of all the three layers of TRC were analysed on GC-ITD based on the chromatographic parameters described earlier showed the presence of 17 prominent peaks. In addition to these compounds, presences of TMB were made by a separate method. In the present ITD scan, 40 to 600 amu range was used. All the 17 compounds fall in the mass range of 88 to 281 amu. Separation of data among MICT, DMIC and 2,4-dione was carried out by same software techniques.

The major compound MICT was eluting at 6.05 min with normalised area percent of 58.00, it showed the base ion m/z 58 followed by its molecular ion peak m/z (M)+ 171 and other significant ions m/z 143, 70, 44, 85 and 113, with the total intensity of 4046. DMI, the second major compound, eluting at 6.24 min comprising 25 normalised area percent, m/z 58 was the base peak followed by m/z (M)+ peak 157, and others m/z 70, 43, 128, 129, 100 and 85. Total intensity of the spectrum was 2844. The third major compound 2,4-dione has base peak m/z 42 ion followed by m/z 156, 158 (M+1)+, 99, 56, 72 and 113. The total intensity of the spectrum was 190951 and eluted at 6.53 min accounting 10.28 normalised area%. The methyl ureas showed a closed relationship on DB-5 column with a slight separation. TMU was eluting at 4.23 min and comprised 4.1 normalised area% and showing fragments base ion m/z 44 followed by 72, 102 (M)+, 85, 103 and 42 ions with total intensity of 55467, whereas DMU was closely eluting at 4.25 min with normalised area percent of 2.65 and on its break down gives m/z 58 the base ion followed by m/z 88 (M)+, 89, 44, 83 and 42 ions with the total intensity of 1851.

Twelve new compounds were detected in the analysis, they accounted 0.1 to 2.0 percent shown in table 8.1 (Rao et al., 1991). The literature so far published did not show the presence of any of these compounds.

Table 8.1. Unidentified TRC Chemical

S.No.	Peak No.	Highest m/z	Principal mass fragments m/z(decreasing order of concentration)	Area %
1.	3	142	42, 56, 58, 127, 142, 128, 70, 100	>0.5
2.	7	156	42,156, 56, 58, 157, 57, 70, 99	>0.5
3.	8	163	58, 56, 163, 42, 134, 157, 44, 70	>0.5
4.	9	162	58, 56, 162, 44,161, 157, 42, 70	>0.5
5.	10	177	44, 58, 56, 43, 157, 42, 177, 70	>0.1
6.	11	196	175, 44, 176, 42, 56, 196, 71, 57	>0.1
7.	12	211	70, 56, 42, 44, 57, 175, 176, 157, 211	>0.1
8.	13	182	44, 56, 58, 43, 157, 42, 70, 176	>0.1
9.	14	181	44, 42, 56, 58, 57, 70. 157, 192	>0.1
10.	15	279	149, 41, 150, 205, 42, 223, 151, 76	>0.1
11.	16	269	70, 155, 56, 42, 69, 183, 241, 269	>0.2
12.	17	267	56, 42, 44, 70, 69, 155, 267, 195	>0.1

Separation of TRCs: To understand the chemical nature of the toxic cloud that descended on the city, it was necessary to study each TRC. The analysis resulted in identification of presence of 12 unidentified TRC. An attempt was made to separate, identify and characterise individual compounds using specific analytical systems like TLC, GC, MS, NMR, IR techniques. Separation and isolation of the TRC was initially tried by solvent extraction procedure. The compounds in the TRC were the products of an exothermic chain reaction and these may have similar physical and chemical properties, hence, it was quite tedious to get good purity for each compound. Therefore, column chromatography, an efficient technique for isolating the compounds, was selected.

Confirmation of Reference and Isolated Compounds of TRC: The reference compounds used for this study like MICT, DMIC, 2,4-dione and TMU were isolated from TRC following the procedure described earlier, whereas TMB, MICT and TMA were synthesised during the study.

Pure MIC used for the experiments, was received from DRDE, Gwalior, its mass spectrum was obtained on GC-ITD. A sharp peak at 247 sec retention time was obtained. Mass fragments (m/z 19, 28, 29, 44, 56, 57, 58) clearly confirmed that

the compound was methyl isocyanate on matching with NBS library, which gave a fit value of 919. TMB was synthesised successfully by reacting urea (NH_2CONH_2) with MIC and subsequent Pyrolysis at 100°C in glass sealed tubes for one hour. TMB was crystallized with hot benzene. MICT was synthesised by pyrolysis of 1,3-DMU and trimerisation of MIC at elevated temperature. TMU was synthesised by methylating MMU with MIC in aqueous solution. The identity of isolated and synthesised compounds was made by studying either melting point, ultraviolet absorption, GC-NPD, GC-MS analyses and/or nuclear magnetic resonance spectra and comparing with available pure compounds.

MICT isolated was compared for melting point (Table 8.2), ultraviolet spectrum and mass fragmentation to that of pure compound obtained from DRDE, Gwalior, confirmed by NMR 1H and infrared spectra. The melting point of the isolate ranged between 175-177°C and agreed to that of pure standard. The ultraviolet spectrum between 200-400 nm showed absorption maxima at 220.5 nm in methyl alcohol and similar spectrum was observed with standard MICT. The 70 eV electron ionisation (EI) mass spectrum gave significant peaks at m/z 58, 171(M+), 143, 70, 44, 85 and 113 ions with sound to noise ratio of 59.6 and matched with National Bureau of Standards (NBS) library as well as standard with a fit value of 922 out of 1000 counts. DMIC isolate melted between 208-210°C and gave 99.9% purity on GC-ITD and showed principal mass fragmentation peaks at m/z 58, 157, 70, 43, 128, 100 and 85 ions and matched with that of literature value (D'Silva et al., 1986). Its UV spectrum gave maxima at 214 nm. The purified 2,4-dione showed a low melting point of 80-82°C, the literature values were slightly varied. D'Silva et al., 1986 observed it as 87-92°C and Etienne and Bonte, 1974 reported it as 95°C. The principal mass fragments were at m/z 42, 156, 56, 99 and 113 ions. The UV spectrum showed its maxima at 237 nm. TMU and TMB, gave melting points 72-73°C and 119-122°C respectively and these values were in agreement to that of literature values (D'Silva et al., 1986) reported. The principal mass fragmentation of TMU were m/z 44, 72, 102, 58, 85 and 89 and gave a fit value of 925 to that reference NBS library search. Whereas TMB gave mass fragments at m/z 58, 88, 44, 145, 115 and UV spectrum gave maxima at 218.5 nm.

Table 8.2. Melting points of Standard Compounds

S. No.	Compound	Melting Point (in ° C)
1.	MICT	175 – 177
2.	DMI	208 – 210
3.	2,4-dione	80 – 82
4.	m/z 269 (Spiro)	218 – 221
5.	m/z 279	195 – 200
6.	Trimethyl Biuret	119 – 122
7.	Dimethyl Urea	101 – 104
8.	Trimethyl Urea	72 – 73

Physicochemical Properties of Unidentified Compounds: Out of those 12 unidentified compounds, peak 15 and peak 16 of GC-ITD analyses, i.e., compounds m/z 279 and m/z 269 were isolated and purified by method developed during study. Colour, appearance, melting points, ultraviolet absorption spectra and EI 70 eV mass fragmentation spectra were recorded. To get more details on the structure of these compounds, studies were carried out at IIT, Madras and IICT, Hyderabad using High resolution mass spectrometer and infrared spectrophotometer and nuclear magnetic resonance spectrometer.

Compound m/z 269 was colourless and crystalline in appearance, its melting point was 220-224°C and absorption maxima at 215 nm in the range of 200 to 400 nm UV spectrum. The principal mass fragments of EI 70 eV were at m/z 56, 155, 183, 42, 211, 241 and 269 (M)+. The FTIR spectrum showed three significant absorption peaks between 1650 to 1750 cm^{-1}, indicating presence of carbonyl groups and other principal infrared peaks at 3000, 1500 and 1050 cm^{-1}. The NMR spectrum was obtained from IICT, Hyderabad on model Gemini 200, 1^H, 13^C NMR. The high resolution mass spectrum data was obtained at 70 eV and 20 eV from IICT, Hyderabad. At 20 eV EI mass fragmentation a significant increase in intensity was observed for m/z 155, 183, 241 and 269 ions and a decrease for m/z 70 and 28 ions. Some ions like m/z 56, 43, 99 disappeared from the spectrum. The relevant data is shown in Table 8.2. Also a high resolution mass spectrum was also recorded at IIT, Madras. The study revealed that this compound could be formed by the reaction of five molecules of methyl isocyanate (Chandra et al., 1994).

Compound m/z 279 isolated from TRC was slight brownish in colour and fine crystals in nature. Its melting point

was 195-200°C, showed two significant absorption peaks at 238 nm, the major, and at 272 nm in the UV light of 200 to 400 nm range. The mass spectrum recorded on 20 eV and 70 eV at IICT, Hyderabad.

We wish to place on record our sincere thanks for the help given by Prof (Dr) DV Ramana of IIT, Madras for some of the spectral analysis of compound 269. At the instance of Dr S Varadarajan further work was carried out at IICT, Hyderabad. Grateful thanks are due to Dr AV Rama Rao and Dr M Vairamani for elucidating the structure of compound 269 and charaterising it as a Spiro compound, although not a nitrile.

Spectrophotometric Cross Verification of Any Emission or Liberation of Cyanide at Ambient Temperatures: Presence of cyanide was studied at ambient temperatures by taking approximately 0.5 g TRC of all the three layers in micro diffusion dishes and also directly in hermetically closed test tubes. In the experiment 2 ml of 0.1 N NaOH solution was used as trapping solution. The entire 2 ml of NaOH solution was used for estimation of cyanide. At room temperature a faint pink colour was observed after the addition of chromogenic reagent benzidine-pyridine. The layer 3 had about 100 µg percent but definite colour development indicate presence of cyanide ions in the TRC.

Water Elutable Cyanide in TRCs: Approximately 0.5 g of the TRC layers 1, 2 and 3 were used for studying water elutable cyanide in TRC following the addition of reagents as described earlier. Calibration curve of standard potassium cyanide was plotted in the concentration 75 ng percent to 1200 ng percent and it was linear. The cyanide ion was recorded 400, 500 and 600 ng percent, for layer 1, layer 2 and layer 3 respectively, with the mean value of 500 ng percent, at ambient temperature.

The coloured complexes were read by DU-64 spectrophotometer as per the procedure and the absorption spectra of standard KCN and TRC was recorded between 350 to 650 nm. In case of pyridine- benzidine method the reddish coloured complex gave absorption maxima at 530 nm. Similar absorption spectrum was obtained for tank effluent indicating definite presence of cyanide.

Studies on Pyrolysis of TRC for Generation of Cyanide: The potential for liberation of cyanide by suspected nitrile derivatives of MIC was explored further. The TRC from the three different layers was pyrolysed in the glass tubes closed with teflon tape and processed according to the procedure showed by cyanide peak on Super-Q teflon column under FID. A clear peak of cyanide was observed at 4.2 min corresponding to that of reference cyanide. The presence of cyanide peak was again confirmed by peak shift at 125°C oven temperature changing the reference retention time to 7.15 min. The head space of the pyrolysed TRC gave a clear peak corresponding to that of cyanide at 7.20 min. Apart from cyanide, many other gaseous compounds were also observed in the head space of TRC. Interestingly, initial cyanide concentration that could not be detected on FID at ambient temperature, could be detected after pyrolysis at 300°C and above pyrolysis temperatures. The cyanide peak increased proportionately with increase in pyrolysis temperature (Table 8.3), determined spectrophotometrically. It may be observed that there is no significant difference among the three layers in production of cyanide. Upto 400°C temperature the mean percentage remained less than one, whereas a massive production was observed at 500°C accounting for 7 to 15 percent. At 600°C one fourth of the TRC was converted to cyanide. It appears cyanide was one of the major end-product of pyrolysis (Chandra et al., 1994b).

MICT, DMU and DMI were pyrolysed at 500ºC to evaluate significance of temperature in generating cyanide. It was observed that MICT and DMI had potentiality in generating HCN. MICT gave a mean value 12.5 percent production over 10.1 percent of mean value obtained for all the three layers. It indicates MICT is the major contributor in generation of cyanide. DMI gave 8.4 percent, whereas DMU did not show generation of any significant amount.

Table 8.3. Pyrolysis of TRCs (CN production in %)

Temp	Layer 1				Layer 2				Layer 3			
° C	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
300	0.024	0.029	0.031	0.028	0.020	0.029	0.026	0.025	0.025	0.034	0.031	0.030
350	0.140	0.136	0.156	0.144	0.174	0.193	0.185	0.184	0.141	0.146	0.163	0.150
400	0.68	0.81	0.76	0.75	0.094	0.97	0.82	0.91	0.50	0.35	0.41	0.42
500	6.5	7.2	7.9	7.2	9.6	10.2	8.7	9.5	11.4	13.8	15.3	13.5
600	25.6	20.2	18.7	21.5	26.0	30.0	21.4	25.8	27.6	31.8	37.8	32.4

Pyrolysis was carried out in sealed corning glass tubes (30 cm x 1 cm internal diameter). About 100 mg of the sample was pyrolysed at various temperatures such as 100, 200, 250, 300, 350, 400, 450, 500 and 600 degree Celsius. The sample include all three layers of TRC, MICT, DMIC, 2,4-dione, m/z 269 amu, m/z 279 amu and DMU. At each temperature every sample was pyrolysed for 1 h and after the pyrolysis these tubes were cooled at room temperature. The tubes were broken from the tip in such a manner that they do not break more than 2 mm. Chloroform has been found to be the best solvent, 5 ml was put in the tube, shaken it thoroughly to dissolve the matter completely. Later the solution was transferred into stoppered test tubes and analysed on by GC-MS using the method as described earlier for its constituents.

The residue left out in the pyrolysis tubes after treating the TRC at various temperatures was analysed on GC-FID using OV- 17 GLT packed column as per the procedure described earlier and on GC- ITD using DB-5 capillary column following the procedure earlier given. The pyrolysis temperatures used were 50°C, 100°C, 150°C, 200, 250°C, 300°C, 325°C, 350°C, 375°C, 400°C, 500°C and 600°C.

It was interesting to note that there was no significant change in the qualitative picture upto 300°C for the chloroform soluble fraction either on GC-FID or on GC-ITD. However, GC-FID study showed degradation of methyl ureas to a great extent and MICT, DMI and dione to a lesser extent. The quantitative change of methyl ureas was prominent between 200-250°C. At 300 & 350°C, only MICT peak was present and others disappeared from the chromatogram (Table 8.4). It is apparent that all the compounds have the greater proclivity for increased concentration with higher temperature and secondly with greater evidence of cyanogenesis. This only indicates the potential hazard or risk of cyanogenesis.

Table 8.4. GC-ITD Studies on TRC Composition at Different Temperatures (Composition in Normalised Area Percent)

Compound	Temperature in °C									
	Ambient	**200**	**250**	**300**	**325**	**350**	**375**	**400**	**500**	**600**
1. TMU	4.10	2.69	2.30	4.23	5.09	7.99	-	-	-	-
2. DMU	2.65	0.92	0.87	0.89	-	-	-	-	-	-
3.M/Z 142	0.32	1.09	1.13	1.13	2.33	3.07	4.45	-	-	-
4.MICT	**58.00**	**3.69**	**72.82**	**83.58**	**28.24**	**59.23**	**95.55**	**100**	**100**	**-**
5.DMI	23.55	3.69	14.40	3.28	4.87	-	-	-	-	-
6. Dione	10.28	6.72	7.31	6.05	7.20	5.02	-	-	-	-
7. M/Z 182	0.02	-	-	-	14.51	3.65	-	-	-	-
8. M/Z 211	0.06	-	-	-	34.04	21.01	-	-	-	-
9. M/Z 269	0.23	1.18	1.14	0.81	-	-	-	-	-	-

With a view to infer on the possible co-liberation of cyanide at the time of formation of the solid residues in the tank, the same was subjected to graded pyrolysis. At elevated temperatures above 300°C, on pyrolysis, release of cyanide was observed from the TRC. Its percentage increases as the pyrolysis temperature is raised (Table 8.3). Kinetic studies of significant compounds were performed on GC-FID and GC-ITD gave similar results in the change of the composition of TRC studies. These results compared well with the spectro-photometric analyses. Dimethyl urea and m/z 269 compound disappeared after the temperature 300°C, whereas TMU, and 2,4-dione after 350°C, sudden appearance of m/z 162 and m/z 211 were observed at 325°C and these continued till 350°C. Presence of DMIC was observed till 350°C and m/z 142 upto 375°C. Only MICT was present upto the temperature 500°C. After 500°C none of the TRC was indicated but for cyanide.

It could be inferred that at the time of the event most of the free cyanide was liberated. The temperature would have reached beyond 300°C. Though MIC on pyrolysis liberates more than 3% HCN even at temperature of 200°C (DRDE, 1985).

Identification of TRCs: To elucidate of above nitrile compounds extra efforts was made to identify the several TRCs detected so far. The experiences of other interested groups in analysis of TRC are presented in the composite Table 8.5. Studies carried out by Varadarajan et al., (1985), Union Carbide Corporation (1985) and D'Silva et al., (1986) show

presence of almost similar product composition of the TRCs. The formation of these compounds was very well discussed by them.

As stated elsewhere, in the course of the search for cyanide yielding chemicals, independently or in complex formation, in the blood and viscera of acute-victims, quite a few compounds were detected. With a view to establish their possible origin, it was considered to look for similar TRC. It would not only validate the observations but also throw fresh light on the toxicological aspects.

It was observed from the GC-ITD that the number of compounds present in the chloroform solution of the TRC were eighteen, which does not include the MMA, DMA, TMA and HCN. Amines have been identified on GC-FID and cyanide has been estimated spectrophotometrically. The mass fragments (m/z) of these compounds revealed that six compounds are similar to those as has been reported by Varadarajan et al., 1985, UCC, 1985 and D'Silva et al., 1986, while other twelve compounds were new, not reported so far (Rao et al., 1991). The relative percentage of these six compounds also varied from the other two reports. It may be because of heterogeneity of the TRCs. As a result of the combined TRC studies it is apparent that a total of 28 components were reported (Table 8.5).

When the run on ITD was continued further more compounds showed their presence. The concentration was low, but it is not the quantity but the toxicology of those compounds more so in disguised synergistic effects, is a point for

Table 8.5. Chemical Compounds of Tank 610 Residue

S.N	Name of the Compound	MW amu	This Study (22 Compounds) GC-MS; FID & UV visual Spectro-photometry %	Varadarajan etal., 1985 (13) Compounds %	UCC 1985 (15 Compounds) %	D'Silva et al., (13) Compounds %
1	Methyl Isocyanate (MIC)	57	-	-	0.2-1	-
2	Trimethyl Isocyanurate (MICT)	171	50-70	55.71	40-55	}
3	Dimethyl Isocyanurate (DMI)	157	8-15	21.42	13-20	} 70
4	Trimethyl Perhydro 2,4.dione (Dione)	157	10-20	3.13	5-7	}
5	Dimethyl urea (DMU)	88	0.05-0.5	1.29	1-2	}
6	Trimethyl Urea (TMU)	102	4-6	1.52	2-4	} Varying
7	Trimethyl Amine (TMA)	59	2-3	3.38	3-4	} Propor-
8	Dimethyl Amine (DMA)	45	1-2	1.97	2	} tions
9	Monomethyl Amine (MMA)	31	1-2	1.02	1-1.5	}
10	Trimethyl Biuret (TMB)	145	0.5-1	0.94	4.8	}
11	Tetramethyl Biuret (TRMB)	159	-	Traces	3.6	} 4-8
12	Chloroform (CHCl3)	119	-	-	0.4-1.5	1.5
13	Water	18	-	-	2	-
14	Chloride (Cl)	-	-	4.33	2-3	Positive
15	Metallicions (Fe, Cr, Ni, Ca, etc)	-	-	0.2-0.9	0.18-0.28	0.08-0.3
16	**Cyanide (CN)**	**-**	**0.05**	**negative**	**negative**	**negative**
17	Spiro Compound	269	-	-	-	-
18	Unknown Compound	279	2-5	-	-	-
19	Unknown Compound	142	>0.5	-	-	-
20	Unknown Compound	163	>0.5	-	-	-
21	Unknown Compound	162	>0.5	-	-	-
22	Unknown Compound	267	>0.1	-	-	-
23	Unknown Compound	196	>0.1	-	-	-
24	Unknown Compound	211	>0.1	-	-	-
25	Unknown Compound	182	>0.1	-	-	-
26	Unknown Compound	281	>0.1	-	-	-
27	Unknown Compound	156	>0.1	-	-	-
28	Unknown Compound	177	>0.1	-	-	-

consideration and matter to be investigated. The greatest difficulty is that the toxicology of even known compounds is not established. Presence of additional twelve chemical compounds, although in less quantities, was detected on GC-MS (Rao et al., 1991). These compounds are still unknown in their chemical formula and structure, whose molecular weight was established by their respective mass spectra. The added feature or facility of obtaining the electron ionisation spectrum on a fractional submolecular basis provided further clues to the interpretation of the possible structure of each compound. It is fully realized that it may not be as complete or convincing as NMR and FT-IR techniques. Initial studies on GC-FID did not reveal presence of most of these compounds, only peaks at 11.58 min and 18.05 min were observed and identified later according to the study on GC-ITD as unidentified compounds m/z 279 and m/z 269. Why the run was cut short in the studies of Varadarajan et al. (1985) and UCC (1985), is not understood. It could be argued that these compounds labeled by this study as unknown compounds, were of less quantity.

Among the eighteen TRC detected on GC- ITD, compounds MICT, DMIC, 2,4-dione, unidentified compounds m/z 269 and m/z 279 were of greater importance, because initial qualitative studies carried out on GC-ITD confirmed the presence of these compounds in the body tissues of aerosol exposed persons (Rao et al., 1991; Chandra et al., 1991; Chandra et al., 1994a; Saraf et al., 1995). These compounds may be adhering for a longer time in the body tissues and altering the normal mechanism of the body system. It is possible that some of these might explain better and correlate with the general symptoms of the aerosol victims after knowing the structure of these compounds and the properties of functional groups therein. Therefore, greater emphasis is given to these compounds. Out of these five compounds, first four compounds are known by their chemical structures and formulae. Some toxicological studies of MICT on animals were carried out at DRDE, Gwalior, their results show mortality of higher percent (Ramachandran, 1991), yet no toxicological data have been published on these compounds.

The unidentified compounds m/z 269 and m/z 279 are high molecular weight compounds amongst all the species found in the TRC. To understand their chemical behavior it was necessary, first of all, to establish the chemical formula and structure. Attempts were made to gather more structural information by analyzing them on IR, NMR and high resolution mass spectrometer. Since these compounds were not available in pure form, it was very difficult to establish their structures. Hence, these compounds remain to be further isolated and purified in a sufficient quantity. Owing to the reasons that these compounds are present in the TRC below 0.2 percent concentration, solvent extraction procedure could not be employed. Various column chromatographic methods have been adopted to isolate these two unidentified compounds. In this process it was important to remove major compounds, i.e., MICT, DMIC, 2,4-dione and ureas. The unidentified compounds m/z 269 and m/z 279 isolated from TRC by giving yields 0.110 g and 0.020 g respectively. Thereafter, the IR, NMR and High resolution mass spectra were recorded at IIT, Madras with active cooperation of Dr. D.V. Ramana, and through courtesy of the Director, Dr. Rama Rao and active cooperation of Dr. M. Vairamani of IICT, Hyderabad.

Spectral Analyses of the Unidentified Compounds: The analysis by ITD (MS) gives reconstructed ion chromatogram (RIC) of the sample. Electron ionisation (EI) occurs when the sample is in the gaseous state. It is the function of mass analyzer to sort ions out and separate them according to their m/z, hence the mass spectrum obtained is the reconstructed ion chromatogram in ionized state of the parent (molecular) with its daughter ions, this usually referred as m/z value (Saferstein, 1982).

The molecular weights (m/z values) and the electron impact spectra of the as yet unidentified compounds were taken into consideration in speculating the possible routes of formation of each compound. Pyrolysis of methyl isocyanate results in the initial formation of hydrogen cyanide, which subsequently forms "Simple", "Minor", "Major", "Compound Adduct" between one or two molecules of MIC and HCN or other products of thermal decomposition. It may be recalled that such a possibility has been entertained from the year 1927 onwards as per oft-quoted references to the paper of Slotta and Tschesche and subsequently by Patoon, 1967, even prior to the demonstration of an adduct following the "Major and Minor routes" of decomposition demonstrated by Blake and Ijadi- Maghsoodi, (1982). As suggested by Dr. Sriramachari, (1990) there is also a likelihood of formation of more "Complex Adducts" comprising of MIC, HCN and MA, acetonitrile, etc. which were possibly generated during the pyrolysis of MIC under the conditions of the run-away reaction in Bhopal.

On the basis of the Mol Weights and the m/z values of constituent molecular sub-units, an attempt was made towards structural configuration of each one of the new compounds, based on the Major or Minor Routes of thermal

decomposition of MIC described by Blake and Ijadi-Maghsoodi. Pursuing the Major Route, Sriramachari configured tentatively the structural patterns corresponding to their molecular weights, Likewise, Dr. Varadarajan reached similar conclusion taking the Minor route (personal communication). Although none of them were conclusive, some of the postulations are enumerated vide ***Annexure 8.1*** to keep the record straight.

II. Presence of TRCs in Early Autopsy Tissues

Preserved autopsy tissues having history of toxic gas exposure were analysed along with appropriate 'Controls' for the presence of TRCs by GC-ITD technique. TRCs were used as a reference material to establish the nexus between the array of chemicals in the involved Tank 610-E residue and human tissues.

Among the 20 cases of 3rd December 1984 which were positive for TRC, 15 of them contained MICT, 8 cases showed DMIT, 6 cases with Compound m/z 279, 3 cases with Compound m/z 269 and only one case showed the presence of 2,4-dione in blood sample. Two lung tissue samples collected on the same day showed MICT, in addition to m/z 279; and only one sample contained DMIT. The other tissues analysed in this group, i.e., brain and liver did not show any of these compounds.

Among 16 cases of 4th December 1984 collection, 10 were positive for TRCs, all these 10 blood samples contained MICT, apart from DMIT in 3 cases, m/z 279 in 2 cases and m/z 269 in one case.

Out of 25 cases of late December 1984, 61 samples were analysed. Out of the 4 samples, 2 were blood and 2 lung tissues, all these were positive for MICT, but one blood sample contained DMIT in addition, whereas m/z 279 showed its presence in one lung tissue (Table 7.3).

Out of the 6 positive cases of 1985, apart from MICT, compound m/z 279 was seen in 1 sample each of blood as well as of spleen. It was interesting to note that none of the 24 clinical blood samples of 1985 were positive for TRCs. The presence of MICT was found in 5 of the 28 autopsy blood samples of 1985. The same Table also shows that none of the 8 samples of 5 cases of placenta and cord of 1985 were positive for TRCs. As shown in the previous section, 150 samples from 40 autopsies during the period 1986-90 were negative for any of the TRCs (Table 7.4 and 7.5).

Quantitative Analysis of MICT: Apart from the near presence of successful attempts were made to estimate the compounds quantitatively. The MICT that was eluting at 6.07 min on DB- 5 column was studied in detail. A linear response was obtained between 10 to 80 ng on ITD with a minimum detection limit of 5 ng. The same calibration graph was used for quantitation of the blood samples allowing a tolerance limit of 10%. A linear variation was observed in the concentration of MICT present in the blood samples of first two days. The concentration on the 3rd December 1984 ranged from 0.0176 to 284.75 µg/ml and on 4th December it ranged from 0.009 to 148.55 µg/ml, whereas, the rest of the samples collected subsequently did not show much variation. The mean value on the first day of collection was 7.4986 µg/ml with a standard deviation of 4.1471, on the second day the mean value was 0.6161 µg/ml and standard deviation was 0.8446. One sample each of 3rd and 4th December were observed exceptionally high values i.e. 284.7549 µg/ml and 148.556 µg/ml respectively. The concentration decreased within a short time giving a value of 0.2162 ìg/ml in the rest of December 1984 period (Table 8.6).

Table 8.6. MICT concentration in 1984 and 1985 autopsy blood samples in µg/ml.

Date	Case	Positive MICT	Percent (%)	Mean ± SD
3-12-84	32	14	42	4.1471 ± 7.4986
4-12-84	15	9	60	0.6161 ± 0.8446
5-12-84 to 31-12-84	12	2	16	0.2162 ± 0.0031
December 1984	**59**	**25**	**40**	**2.5614 ± 5.8343**
1-1-85 to 31-3-85	6	2	33	0.0401 ± 0.5190
1-4-85 to 30-6-85	9	1	11	0.0670
1-7-85 to 30-9-85	9	2	22	0.1172 ± 0.1091
1-10-85 to 31-12-85	4	-	-	-
Total 1985	**28**	**5**	**17**	**0.0646 ± 0.0785**
Total 1984 & 1985	**87**	**30**	**34**	**2.1452 ± 5.3914**

(One sample of 3-12-84 has 284.7549 µg/ml and one sample of 4-12-84 has 148.556 µg/ml, were not included in table).

However, the presence of MICT was seen till the third quarter of 1985. The third quarter of 1985 gave a mean value of 0.1172 µg/ml. From Table 8.8, it may be observed that not much variation in the concentration of MICT in different age groups. A child group showed persistence of TRC in the body for a longer time than Carbamoyl amino acids.

Table 8.7. Sex-wise distribution of MICT in autopsy samples in µg/ml

Date	Male				Female			
	Cases	MICT	%	Mean ± SD	Cases	MICT	%	Mean ± SD
3-12-84	20	8	40	1.0541 ± 2.3431	12	6	50	8.3255 ± 10.0864
4-12-84	12	8	66	0.5113 ± 0.8381	3	1	33	-
5-12-84 to 31-12-84	5	1	20	-	7	1	14	-
Dec 1984	37	17	45	0.7303 ± 1.6805	22	8	36	6.4526 ± 9.2089
1-1-85 to 31-3-85	4	-	-	-	2	2	100	0.0401 ± 0.0519
1-4-85 to 30-6-85	6	1	16	-	3	-	-	-
1-7-85 to 30-9-85	3	1	33	-	6	1	16	-
1-10-85 to 31-12-85	2	-	-	-	2	-	-	-
1985	**15**	**2**	**13**	**0.1005 ± 0.1327**	**13**	**3**	**23**	**0.1361 ± 0.1861**
1984-85	**52**	**19**	**36**	**0.6640 ± 1.5971**	**35**	**11**	**31**	**4.7037 ± 8.2665**

(One sample of 3-12-84 has 284.7549 µg/ml and one sample of 4-12-84 has 148.556 µg/ml, were not included in table).

From the Table 8.7, it was clearly evident that sex factor did not play any role on the concentration of MICT. In both the cases the mean value remained almost same. In late December 1984 the mean value of male was 0.21 mg/ml and in female 2.14 mg/ml. The mean of 85 samples in Table 8.7 also showed similar trend in both the male and female.

Similarly an analysis of age wise distribution of MICT in 1984-85 demonstrated that the levels are relatively higher in the children in the age group of 5-15 years. This anomaly could be explained in terms of increased physical activity of the older children and consequently increase to exposure to the gases (Table 8.8).

Table 8.8. Age wise distribution of MICT in 1984 and 1985 blood samples in µg/ml.

Date	Up to 5 years				More than 5 to less than 15 year				More than 15 years			
	Cases	Positive MICT	%	Mean ± SD	Cases	Positive MICT	%	Mean ± SD	Cases	Positive MICT	%	Mean ± SD
3.12.84	6	4	66	5.8142 ±11.390	7	5	71	5.0410 ±7.7315	19	5	25	1.1995 ±3.9598
4.12.84 1.2240	3	3	100	0.0230 ±0.0119	4	2	50	2.2989 ±0.066	6	8	4	50 ±0.9911
5.12.84 to 31.12.84	3	1	33	0.2184	-	-	-	-	9	1	11	0.2140
December 1984	**12**	**8**	**66**	**2.2908 ±8.065**	**11**	**7**	**63**	**3.6861 ±6.7235**	**36**	**10**	**27**	**1.4707 ±2.7587**
1.1.85 to 31.3.85	4	2	50	0.0401 ±0.0519	1	-	-	-	1	-	-	-
1.4.85 to 30.6.85	4	1	25	0.0067	1	-	-	-	4	-	-	-
1.7.85 to 30.9.85	5	2	40	0.1172 ±0.1091	-	-	-	-	4	-	-	-
1.10.85 to 31.12.85	-	-	-	-	-	-	-	-	4	-	-	-
1985	13	5	38	0.0642 ±0.0785	2	-	-	-	-	-	-	-
1984-85	25	13	52	1.8344 ±6.329	13	7	52	3.6861 ±6.7235	49	10	20	1.4707 ±2.7587

Conclusion

The possible role of TRCs in neo-cyanogenesis, leading to chronic and recurrent cyanide toxicity, assumed great relevance. Therefore, serious efforts were made to characterise their possible contribution to 'Nitrile Compounds'. The Toxicology Project of ICMR successfully demonstrated the presence of a total of 20 Tank Residue Compounds, including the 11 compounds previously reported by UCC and NCL, Pune. While on 'fresh pyrolysis' they exhibited cyanide release, the results were negative at ambient temperature, Thus, apart from evidentiary value, since both the duration and quantity of these compounds present in autopsy tissues were very limited, their potential contribution to 'delayed or recurrent cyanogenesis' appears to be unlikely.

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Structural postulations of Chemical Compounds Corresponding to their molecular weights

Introduction

There could be more than one pathway for arriving at the chemical synthesis of each one of these compounds, thus D'Silva and co-workers (1986) of the Union Carbide Corporation, based on simulation experiments tried to explain the derivation of practically all the compounds contained in the UCC report, without involving the release of hydrogen cyanide. Since it has been a crucial issue in Bhopal, the possibility of its release and simultaneous its interaction with methyl isocyanate or its major or minor route of decomposition on one hand or other possible reactions had to be considered. In evaluating the different pathways, preference is given to those reactions which can readily account for the electron ionisation spectrum as revealed by the Ion Trap Detector system. Incidentally, an attempt is also made to derive even some of the identified compounds such as methyl isocyanate trimer, dimethyl isocyanurate, 2,4-Dione through these alternate pathways viz... formation of adducts between methyl isocyanate and its major pyrolysed derivatives primarily hydrogen cyanide or acetonitrile, methyl amine (methyl carbamic acid). It is specially noteworthy that these alternate pathways also indicate the scope for the liberation of potentially hazardous chemicals like cyanogen, carbon monoxide, mono and dimethyl amines, alkyl dinitrile compounds like malonyl nitrile and cyanohydrins of varying degrees of toxicity. It must also be pointed out that it is possible to derive many of the formulae not only through the "Major route" of pyrolysis of methyl isocyanate and release of hydrogen cyanide as suggested by Blake and Ijadi-Maghsoodi (1982) but also through "Minor route" of formation of dimethyl carbodiimide, as suggested by Dr. S. Varadarajan, (1990) later on. However, in that case the quantitative yield and chemical consumption or utilisation of hydrogen cyanide and formation of the nitriles would be comparatively much less.

Instead, with a view to provide a proper framework or perspective the reaction have been grouped in the following manner by Dr. Sriramachari a co-investigator in the Toxicology project in Bhopal as an extension of the chromatographic and mass fragmentation patterns obtained in this study:

A. Reactions based on Hydrolysis of methyl isocyanate:

(1) $CH_3NCO + H_2O \longrightarrow CH_3NH.COOH$ ...Eqn. 1

(2) $CH_3NCO + H_2O \longrightarrow CH_3NH_2 + CO_2$ ...Eqn. 2

B. Reactions based on Non-Hydrolytic Decomposition of MIC:
(Blake and Ijadi- Maghsoodi, 1982)

(1) Pyrolysis of MIC (Major route)

$CH_3NCO \longrightarrow H_2 + HCN + CO$ ...Eqn. 3

(2) Pyrolysis of MIC (Minor route)

$2CH_3NCO \longrightarrow CH_3.N=C=N.CH_3 + CO_2$ ...Eqn. 4

N-N' Dimethyl Carbodiimide (Mol Wt 70)

C. Additive/Condensation Reactions of MIC with HCN, MMA, Acetonitrile etc:

(1) With HCN:

(a) $CH_3NCO + HCN \longrightarrow CH_3NHCOCN$ ...Eqn. 5

Simple Adduct (Mol Wt 84)

(b) $CH_3NCO + 2HCN$ or $CH_3NHCOCN + HCN$ $\xrightarrow{-H_2O}$ $H_3NC(CN)_2$ or $H_3NC(CN)_2$ ... Eqn. 6

(b) $2CH_3NCO + HCN \longrightarrow CH_3NHCO.CH_3N.COCN$ Major Adduct (Mol Wt 141) ... Eqn. 7

(2) Complex adduct formation by condensation of Simple adduct with methyl amine (methyl carbamic acid).

(a) $CH_3NHCOCN + CH_3NH.COOH \xrightarrow{-CO_2}$ $CH_3NH_2C(CN)(OH)$ ... Eqn. 8

Bis Methyl Amino Cyanohydrin (Mol Wt 115)

(b) The same as above on removal of one molecule of H_2O produces

$CH_3NH_2C\cdot(CN)$

... Eqn. 9

Bis Methyl Amino nitrile radical (Mol Wt 97)

This compound has "open" valencies on both the carbon and nitrogen atoms of a basic acetonitrile derivative which can readily participate in cyclotrimerisation reaction.

(3) Complex adduct formation by condensation of simple adduct and carbamic acid with acetonitrile formed by interaction of HCN and the alkyl halide, chloroform, which is believed to be present in the tank 610 in sufficient, if not abundant quantities.

$CH_3NH_2C(CN)(OH)$ Eqn. 8 $+CH_3CN$ Acelonitrile $\xrightarrow{-H_2O}$ $CH_3NH_2C(CN)CH_2CN$

Bis Methyl Amino Succinonitrile less Dimethyl Amino ... Eqn. 10

$NHC(CN)CH_2CN$

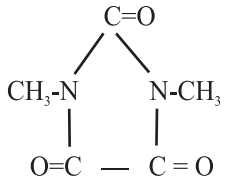
... Eqn. 11

(Mol Wt 93).

This compound has "open" valencies on both the carbon and nitrogen atoms of an amino succino-nitrile derivatives, which can readily participate in a cyclotrimerisation reaction, with one Nitrile molecule on a side chain (alkyl cyanide) and another on the ring structure (aryl cyanide).]"

1. M/z 142

Looking to the mass fragmentation pattern of peak 3 that was eluting at 4.56 min, it may be said that this was similar in structure to that of methyl isocyanate trimer m/z 171. The methyl isocyanate trimer gave a mass spectrum with principal fragments at 171, 143, 128, 113, 85, 70 and 58 whereas peak 3 m/z 142 gave fragments at 142, 128, 100, 70 and 58. This m/z 142 fragment from methyl isocyanate trimer was an ion form after loosing one methylazine (=N-CH_3) group, its probable structure could be :



Its nomenclature can be given as 1,3,-dimethyl- (1,3,-diazine)- 2,4,5-trione and molecular formula would be $C_5H_6N_2O_3$.

Patoon (1967) is reported to have suggested the addition/ condensation reactions of MIC with HCN

$$CH_3NCO + HCN \longrightarrow CH_3\text{-}NHCO.CH_3N.COCN$$

Major adduct (Mol wt 141)

2. M/z 269

This compound is of paramount importance because of its presence in human system of the aerosol exposed victims (Chandra et al., 1994a). This compound was separated from the tank residue in sufficient amount and was eluting at 18.5 min on GC-FID and on GC-ITD at 14.29 min, its mass, UV, 1H NMR, IR spectra were recorded at other laboratories.

*IR cm^{-1} : 740, 750, 1695, 1710, 1740 and 1795.

*^{1}H NMR ppm : 2.82 (S, 9H), 3.15 (S, 3H), 3.26 (S, 3H)

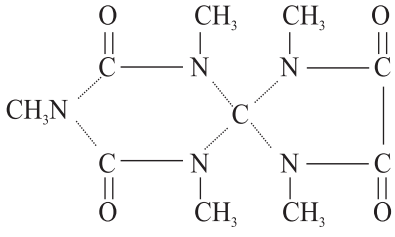
^{13}C NMR ppm : 22.7, 24.2, 28.2, 86, 150, 153.2 and 166.5.

Mass m/z : 269 (M) +, 241, 211, 183, 155, 83, 70 and 56.

elemental analysis:C=45.73,H=5.231,and N=25.09.

Based on these studies its structure could be written as shown.

From the structure its chemical formula would be, $C_{10}H_{15}N_5O_4$. It is a spiro compound. Earlier studies were made on



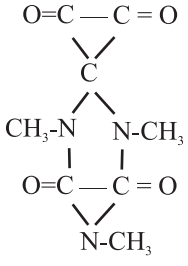
$C_{10}H_{15}N_5O_4$

1,3,6,8,10-pentaazaspiro-[5]-decan--2,4,7,9-tetranon

its formation by combining five molecules of methyl isocyanate in the presence of ruthenium or triethylsilane as catalyst (G-Suss-Funk et al., 1983; 1985; 1986). Its toxicological properties are not reported.

3. M/z 211

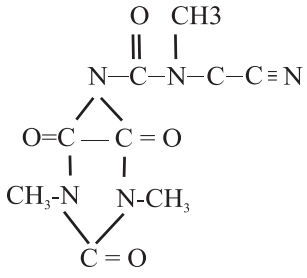
This compound is peak 12 of GC-ITD run, eluting at 9.44 min showed significant m/z at 211, 183, 155, 56. Based on the mass fragmentation pattern of this unidentified compound, its structure could be drawn as:



Its chemical formula might be given as $C_8H_9N_3O_4$. The mass fragmentation pattern of compound m/z 211 and m/z 269 has more similarities and hence it may be said that compound m/z 211 is a precursor or a by product of compound m/z 269.

4. M/z 267

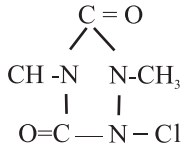
This compound is peak number 17, eluting at 14.48 min on GC-ITD run of tank residue and could be formed as a minor adduct of some reactions, evaluation of its fragmentation pattern of mass spectrometer, it may be assumed that at elevated temperature methyl isocyanate trimer reacts further with methyl isocyanate and hydrogen cyanide and formed this compound. Its probable structure could be given as shown.



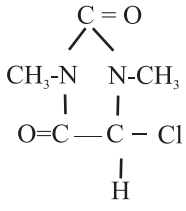
$C_9H_9N_5O_5$

7. M/z 163 AND 8. M/z 162

These compounds, peak 8 and peak 9 of GC-ITD run, m/z 163 and 162 eluting at 7.47 min and 8.07 min could be similar species of one compound. These are present in almost equal mixture. According to their fragmentation pattern their structure could be given as:



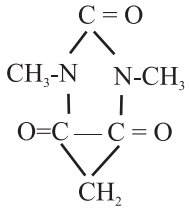
m/z 163
($C_4H_6N_3O_2Cl$)



m/z 162
($C_5H_7N_2O_2Cl$)

6. M/z 156

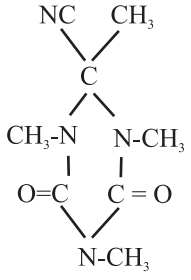
The next compound in the series of unknown compounds is having m/z 156, this compound is eluting at 7.00 min on GC-ITD run, right after the 2,4-dione and have the same m/z value i.e. 156. This compound may be a rearranged product of 2,4-dione, its probable structure according to the mass fragmentation pattern could be given as following:



This compound could be formed by replacement of one -N-CH_3 group by methylene (-CH_2) group. This could have the molecular formula $C_6H_8N_2O_3$.

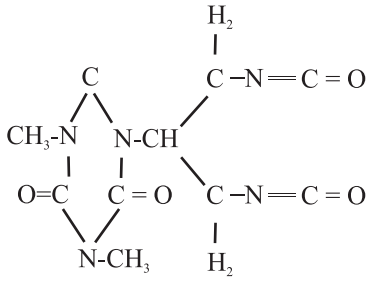
7. M/z 196

The unidentified m/z 196 compound, eluting at 9.35 min of GC-ITD run. According to its mass fragmentation pattern and its molecular structure, its molecular formula could be written as $C_8H_{12}N_4O_2$.

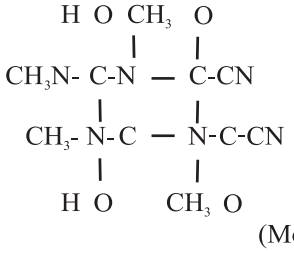


8. M/z 281

This compound has highest m/z value among all the tank residue entities and eluting at 12.45 min. It might be formed by the addition reaction of methyl isocyanate on methyl isocyanate trimer or addition of five methyl isocyanate molecules. This compound could be formed by the condensation of methyl isocyanate and hydrogen cyanide. The electron ionisation spectrum of singlet and doublets of major adduct (i.e. 2MIC + HCN) of molecular weight 141 represented as a 5 membered hetrocyclic dione ring by Patoon (1967) or linear alkyl nitrile by Blake and Ijadi-Maghsoodi (1982) could be explained in either case. Its structures could be drawn as per its mass fragmentation pattern.



It has been suggested by Dr. Sriramachari, (1990) that if the condensation of two molecules of "Major Adduct" in an asymmetrical N-C linkage at either end could also occur in the following manner:

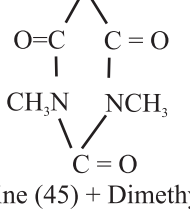


(Mol Wt 282)

In turn such a molecule, may further undergo molecular rearrangements as follows:

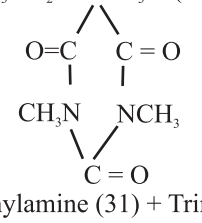
(b) Condensation in the isomeric configuration of the end terminal group in one of the major adduct molecule result in corresponding alterations in the end products.

(a) $(CH_3)_2NH + NH + (CN)_2 + CO$



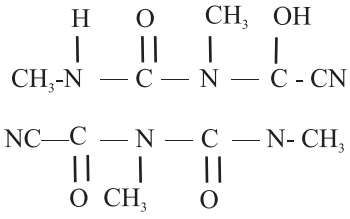
Dimethylamine (45) + Dimethyl Isocyanurate (157) + Cyanogen (52) + Carbon Monoxide (28)

$CH_3NH_2 + NCH_3 + (CN)_2 + CO$



Monomethylamine (31) + Trimcthyl Isocyanurate-MIC Trimer (17) + Cyanogen (52) + Carbon Monoxide (28)

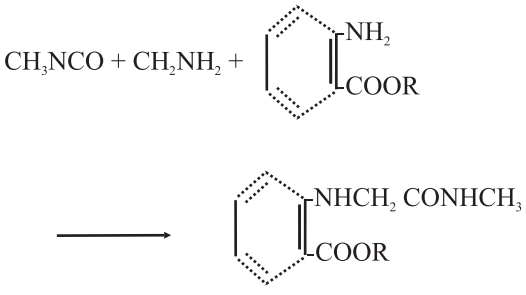
Thus, it is noteworthy that although formation of methyl isocyanate trimer can result from simple polymerisation of methyl isocyanate, which is perhaps the preferred route, it could also be formed as a by-product of condensation of two molecules of the "major adduct", which must have been generated in substantial amounts (Blake and Ijadi-Maghsoodi, 1982).



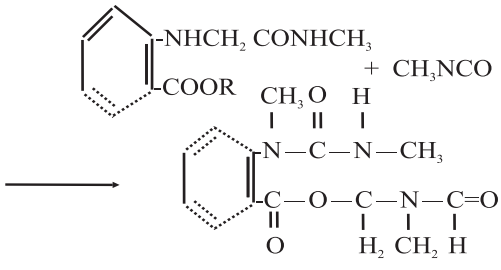
On the other hand in the case of the dimethyl isocyanurate simple condensation of methyl isocyanate and substitution of one methyl group by a hydrogen atom may not be the preferential route. Instead, it is more likely that the condensation is from a pair of major adduct molecules, followed by the liberation of dimethylamine, cyanogen or carbon monoxide and possibly even methyl cyanide. The fragmentation pattern of unidentified compound with m/z 281 corresponding to peak 14 has been examined. It appears to be in accordance with the giant molecule formed by condensation of two molecules of the major adduct.

9. M/z 279

This compound is also having great significance because of its presence in the body tissues of aerosol exposed victims. This is eluting at 13.53 min of GC-ITD run. UCC bulletin F-41443A-7/76 quoted a reference of Capuano and Zander, (1966).



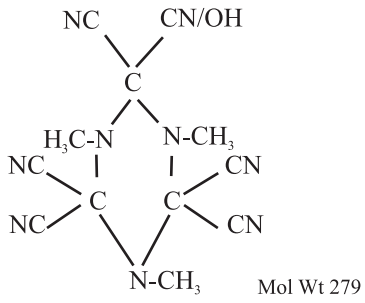
All of these compounds were present in the tank with methyl isocyanate (UCC, 1985), and molecular weight of the resultant product is 222. Methyl isocyanate was available in sufficient amount may be added to the above resultant by giving the unidentified compound m/z 279. Its molecular formula may be $C_{13}H_{17}N_3O_4$.



This unidentified compound may be formed by cyclotrimerisation of equations 6, 9 or 11 can give rise to the following heterocyclic symmetrical methyl triazine or triazine polynitriles (hexa or tri).

(1) Cyclotrimerisation as per Eqn. 6:

The compound exactly corresponds to that of peak 15.

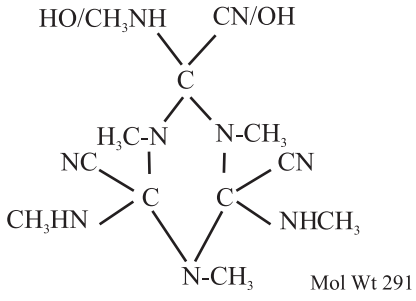


Mol Wt 279

(2) Cyclotrimerisation as per Eqn. 9.

Substitution of one nitrile group by OH group gave a Mol Wt 282 and one Methyl amino group by OH group. A Mol Wt of 278_(279) this is nearest to that of peak 15.

This formula can support the EI of 279/223/205/149 by fragmentation of methyl cyanamide radical (Mol Wt 56), hydroxyl group (17) followed by another methyl cyanamide radical (56) followed by 149 with a Cyano-Trimethyl Triazine residue.

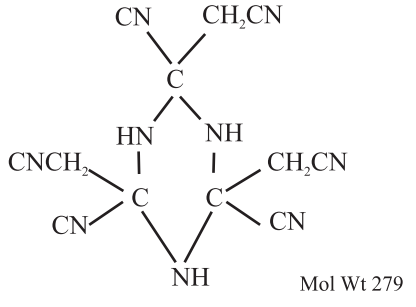


Mol Wt 291

(3) Cyclotrimerisation as per Eqn. 11:

The Compound enactly corresponds to that of peak 15

Being an alkyl aryl polynitrile compound with molecular weight value of 279, and if it fulfills the electron ionisation pattern, it would have been ideal since the alkyl chain based nitrile groups can yield SCN^{-} (thiocyanate) more easily. But it appears to be incapable of sustaining the electron ionisation pattern of 279/223/205/149 etc



Mol Wt 279

However, in either case, the formation of other well known toxic compounds explains that the toxic Material/ Aerosol liberated in the UCC plant in Bhopal was indeed a mixture of several toxic gases and compounds.

CHAPTER

9 SUMMARY

AMIDST the ICMR-supported Research Projects related to Bhopal Gas Disaster **Project 08 entitled 'Pathology & Toxicology',** assumed great importance from several aspects. It involved generation of hitherto unknown and almost new scientific evidence and ensuring the rationale of therapeutic intervention and detoxification. The exhaustive autopsy studies in the acute, sub-acute & chronic phases confirmed the age-old wisdom that the 'dead teach the living', especially when confronted with new and virtually unknown chemical(s). Very early study of 'Externals & Internals at Autopsy' was followed by systematic Histopathological study of representative samples under Light & Electron Microscopy which established the progressive nature of the lesions. Similarly, appropriate short & long-term Experimental studies with single dosage of MIC, the offending chemical and even its aqueous derivatives, Methyl Amine and Di-Methyl Urea, dispelled some of the wrong information circulated by UCC, the manufacturer.

A close follow-up of the initial autopsy findings of **Cherry Red Tissue Discoloration** opened the Pandora's Box, of not just merely Acute, but Delayed and Recurrent Cyanide Toxicity, but the phenomenon of 'Carbamoylation' of end-terminal Amino-&-Sulphydryl Groups, contributed towards an understanding of the underlying 'metabolic lesion' of MIC toxicity. Although these scientific phenomena perhaps seem to be known in the literature, it has been unraveled *de novo* by the Project 08 ICMR. More importantly, the generation of the new toxicological evidence had offset the inadequacy of 'Routine Clinical Laboratory Tests' in a totally new chemical disaster. In this Summary Chapter, an attempt has been made to highlight the several aspects of the Toxicology Project.

At the outset it is worth recalling that the Project 08 is one which was in reality ***multi-institutional*** & ***trans-disciplinary***. People started dying within hours of the ghastly 'Bhopal Gas Tragedy / Disaster', re-designated as 'MIC Aerosol Tragedy' by Late. Prof. Heeresh Chandra. Indeed he planned and immediately initiated the autopsy studies from the afternoon of 3rd December, 1984, with the assistance of entire Medical & Scientific staff of MLI and the Department of Pathology, MGMC, Bhopal. The ICMR team from the Institute of Pathology, New Delhi, actively joined, not only in the conduct of autopsy studies but also undertook corresponding Histopathology, Electron Microscopy & Toxicological investigations. Relevant Toxicological & Experimental studies were carried out in collaboration with wide range of co-investigators / experts drawn from AIIMS, GB Pant Hospital, INMAS and DIPAS, New Delhi and DRDE, Gwalior and suggestions from several other Institutions across the country were sought after.

Overview of Toxicology Issues of BGD

An attempt has been made to focus attention on the several imponderable issues surrounding Bhopal Gas Disaster due to sudden and massive leak of Methyl IsoCyanate leading to death of several thousands of people. There was an inexplicable gap about toxicology MIC; even with regard to the base line data about the physical chemistry of MIC and its polymers or their long-term storage, aqueous reactivity and spectrum of thermal decomposition. It was in 1982, just two years prior to the Bhopal Gas Disaster, that Blake & Izadi–Maghsoodi published their pioneering work on thermal decomposition of MIC, associated with liberation of highly lethal compounds like CO, HCN and the possibility of their forming Nitrile adducts with the original MIC through two distinct pathways or ***major and minor routes***. Apart from probable unawareness of the issues by UCC and even a majority of the world scientists, prior to the BGD, very little seems to be known about Carbamoylation of Valine residues of Hb in the treatment of sickle cell anemia. It is indeed fortuitous that the Toxicology Project of ICMR at MLI, in scientific collaboration with IOP, Delhi & DRDE, Gwalior were the first to draw attention to both the issues of Cyanide Toxicity and Carbamoylation of Valine residue of Hb.

Gross Human Autopsy Studies (External & Internal)

On the day the Bhopal Gas Tragedy occurred, none could have imagined or anticipated its complexity, magnitude or duration. It was indeed fortuitous that right from the very start, Late Prof. Heeresh Chandra serendipitously garnered systematic Medico Legal Evidence including public display of the images of the dead. It is noteworthy that out of 731 bodies received in the month of December 1984, as many as 620 occurred in the first two or three days, strongly suggestive of some 'rapid and acute lethal toxicity'. While External Autopsy findings were recorded in all the 731 bodies, detailed autopsies could be performed in 394 cases during the month of December 1984. Out of them, the Pathology Department of MGI studied Histopathological findings in 40 acute cases, followed by 22 acute cases by IOP. Later, over the next few years, the IOP undertook a comprehensive study of a total of 172 cases of Sub-Acute & Chronic Cases of exposure.

There were some unique patho-gnomonic findings such as widespread conjuctival congestion, nasal and oral frothing and fluid exudation, pinkish discoloration of the bodies and lack of cyanosis. Similarly, 'Cherry Red Discoloration' of blood and viscera was accompanied by edema and hemorrhage. The lungs appeared to be the target organ, followed by brain and other organs to a variable degree.

Histopathological & Experimental Studies

While brief accounts of the pathological findings are presented in the main Chapter, the detailed findings of the sequence of pathological changes are described with appropriate illustrations in the Annexures of the Chapter.

Although many organs were affected, the most prominent findings were in the lungs. In the early series, there was a gross increase in the weight of the lungs, nearly 3 times that of the normal. The entire respiratory tract showed a series of pathological changes. There was intense congestion and denudation of the epithelium of the trachea and the major divisions of the bronchi. There were foci of ulceration. The lungs were heavily water-logged and had the characteristic cherry red colour. Microscopically there were extensive changes such as necrotising bronchiolitis and widespread damage of the lung parenchyma. The major findings were those of acute bronchiolitis, bronchopneumonia, pulmonary hemorrhages and edema, with outpouring of albuminous fluid into the alveoli, pneumonitis and alveolitis. The Histopathological changes in other organs were suggestive of extensive or widespread cerebral edema or swelling, peri-capillary ring hemorrhages, both in the cortex as well as the white matter. The liver in number of cases showed varying degrees of fatty change. The kidneys showed marked congestion in most of the cases and tubular necrosis in some. The gastro-intestinal tract showed mesenteric and sub mucosal hemorrhages, and necrotizing enteritis. The heart muscle showed generalized edema without obvious necrosis.

A subsequent group of autopsies on victims who died during the 8-12 week after the episode revealed less marked but essentially a similar picture of pulmonary changes. But there was no suggestion of any interstitial or parenchymal fibrosis at that stage. The observations of cerebral edema and anoxia were consistently prominent even in the late stage.

Similarly, Experimental Studies were undertaken with a view to dispel UCC's subtle campaign about relatively low toxicity of MIC and its aqueous degradation products. Therefore, a comparatively long-term experimental study of MIC and even its aqueous derivatives, Methyl Amine & Di-Methyl-Urea was carried out, with very interesting results, quite comparable to Human lesions. First, the progression of severe pulmonary edema to chronic fibrosis was confirmed experimentally, following a single exposure to MIC. Detailed accounts of the sequential pathological changes are presented in ***Annexure 4.2***.

Pathophysiology & Inhalation Toxicology

The Toxicological Studies of ICMR clearly established through Direct & Indirect evidence suggestive of not only 'acute cyanide toxicity' but also 'delayed or recurrent cyanide toxicity' due to its thermal decomposition products like HCN and/or 'recurrent cyanide toxicity' through N- & S- Carbamoylation. The possible interplay and mechanisms of each category has been discussed. For a better understanding, the contribution of CO & HCN in BGD has been discussed from chronological perspective of thermal decomposition of MIC (Blake & Ijadi-Maghsoodi) and also the very recent studies on 'Weapons Emissions' in the course of gun firing of apparently unrelated artillery chemicals (Halperin, 2008).

Urinary Thiocyanate & Cyanide Toxicity

The enormous controversies about elevated thiocyanate levels in the urine of exposees was more than resolved by demonstrating higher levels in exposed population as against unexposed controls, especially after administration of NaTS injections to the survivors. In addition to 'clinical relief', the initial rise in the urinary NaSCN levels gradually declined. The rationale of NaTS therapy was further substantiated by elevated urinary NaSCN levels in Double Blind Clinical Trials as well as in clinical patients. The overall elevated levels of 58.0% in early 1985, gradually came down to 7.0% in 1986. A total of 17,527 urine samples have been analysed till October 1987, followed by 1350 samples till June 1988. Further Details are available in the document submitted to Supreme Court and enclosed as ***Annexure 6.2***.

Apart from ensuring the scientific rationale, this study had brought to light the mechanisms of acute, delayed and recurrent cyanide toxicity, probably due to underlying disturbances of cyanide metabolism, during the reversible phases by blockage of Sulphane donors of rhodanese-like enzymes. Similarly, such a mechanism could also be due to trans-carbamoylation between more dynamic Sulfydryl (SH) and end-terminal Amino Groups. ***This is a yet another issue which needs clarification in future.***

Further, the evidence was corroborated by the finding of Elevated Blood & Tissue Cyanide levels of autopsy samples in a total of 123 cases. Indeed the generation of such incontrovertible evidence, more than fulfilled our doubts and expectations. Indeed it is inexplicable as to why and how such a tirade was mounted and sustained against Cyanide Toxicity & NaTS Therapy by different groups of Indian Clinicians, Administrators and even Activists from India & abroad. It is one of the reasons for placing on record the entire evidence of a rare and successful scientific phenomenon following a unique Chemical Disaster.

Blood and Tissue Carbamoylation

Another important contribution of this Toxicicology Report is the demonstration of –binding of MIC to end-terminal amino groups of Hb and tissue proteins, a finding more than of academic interest. Strangely enough, the UCC's campaign against tenability of cyanide toxicity due to hydrolysis of MIC on coming in contact with aqueous surfaces of airways, acted as a stimulus for Toxicology Project 08. It successfully tracked MIC in the blood of Bhopal victims (dead and living). From the very beginning Contrary to UCC campaign, it was successfully demonstrated that MIC crosses alveolar-capillary barrier and is bound to free end-terminal alpha Amino Group of Valine residues. Briefly this process interferes with pick-up of $CO_{2,}$ impairment of Blood & Tissue Oxygenation and the development of a compensatory mechanism of Blood Gas Exchange by elevation of 2-3 DPG levels**.** It is significant that these physiological disturbances are beyond the quantum of binding of MIC to Hb. This unique Indian finding of MIC crossing over alveolar blood barrier was appreciated by Bucher in a personal communication to the Director of DRDE.

Tank Residue Analysis

Lastly, a highly technical and extensive study on Tank Residue Constituents (TRCs) was undertaken, for the detection of other possible 'Cyanide-yielding Nitriles'. Although no nitriles were detected, a total of 21 compounds were demonstrated, including 11 compounds reported previously by UCC and NCL, Pune. In addition, traces of HCN were also demonstrated even after considerable lapse of time. It is important that many of these compounds could be traced in the autopsy tissues especially during the first few days.

There was a lot of delay in timely publication of the data, due to unavoidable issues of Medico Legal Propriety at that time. The available information has been presented in a series of Chapters of this Report, accompanied by appropriate Annexure/s based on 'Published Papers' or as 'Personal Communications'. For the 'record', a brief account was also submitted earlier in 1987 to Dr. CR Krishnamurthy Commission and incorporated in its Final Report.

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