FOETAL TOXICITY OF METHYL ISOCYANATE METABOLITES

by

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This thesis is dedicated

to

my parents

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ABBREVIATIONS

AOAA	Aminooxyacetic acid
CYS	Cysteine
DMA	Dimethylamine
DMU	Dimethylurea
GSH	Glutathione
MIC	Methyl isocyanate
MMA	Monomethylamine
OTC	(-)-2-oxo-4-thiazolidine carboxylic acid
PAGE	Polyacrylamide gel electrophoresis
SMG	S-(N-methylcarbamoyl)glutathione
TMA	Trimethylamine
YS	Yolk sac

ABSTRACT

The foetal toxicity of the methyl isocyanate metabolites trimethylamine (TMA) and S-(N-methylcarbamoyl)glutathione (SMG) was studied in mice. Administration of TMA but not of SMG during pregnancy decreased foetal weight and selectively inhibited the postnatal growth of male offspring. Both TMA and SMG were teratogenic to mouse embryos in culture. TMA caused a dorsal-rostral split in the head and SMG produced distortion and separation of somites; both agents decreased the growth of embryos. The inhibition of embryonic growth by TMA could not be antagonized by antioxidants or growth factors. The embryotoxic effects of SMG could be antagonized by glutathione (GSH). TMA inhibited the incorporation of ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-leucine into their respective macromolecules; SMG inhibited the incorporation of ^{3}H-thymidine. Uptake by the yolk sac placenta and incorporation into embryonic proteins of ^{3}H-leucine-labelled proteins but not of free ^{3}H-leucine was inhibited by TMA at concentrations which did not inhibit lysosomal degradation of proteins. SMG inhibited the uptake and incorporation of both free ^{3}H-leucine and ^{3}H-leucine-labelled proteins. The inhibition of receptor-mediated uptake by SMG could be antagonized by GSH. The data suggest that TMA and SMG produce embryotoxicity mainly by an inhibition of uptake mechanisms of the yolk sac placenta.

RESUMÉ

La toxicité foetale des métabolites du methyl isocyanate, triméthylamine (TMA) et S-(N-methylcarbamoyl)glutathion (SMG) a été étudié chez la souris. L'administration du TMA au cours de la gestation a causé une baisse de poid foetal et a inhibé sélectivement la croissance post-natal chez le mâle. Le TMA et le SMG étaient tératogènes aux embryons de souris en culture. Le TMA produit une fente dorso-rostrale à la tête et le SMG une distortion et séparation des somites; ces deux agents ont ralenti la croissance des embryons. L'inhibition de la croissance embryonaire par le TMA ne pouvait pas être antagonisée par des antioxidants et des facteurs de croissance. Les effets embryotoxiques de la SMG étaient antagonisés par le glutathion (GSH). Le TMA a inhibé l'incorporation de la ^{3}H-thymidine, de l' ^{3}H-uridine et de la ^{3}H-leucine dans leurs macromolécules respectives; le SMG a inhibé l'incorporation de la ^{3}H-thymidine. L'accumulation par la membrane vitelline du placenta et l'incorporation par l'embryon de protéines marquées à la ^{3}H-leucine, et non pas la ^{3}H-leucine libre, était inhibée par le TMA à des concentrations qui n'empêchent pas la dégradation des protéines par les lysosomes. L'accumulation et l'incorporation de protéines marquées à la ^{3}H-leucine et la ^{3}H-leucine libre était inhibée par le SMG. L'inhibition de l'accumulation de SMG était antagonisée par le GSH. Il est conclu que l'effet embryotoxique du TMA et du SMG est produit principalement par l'inhibition des mécanismes de transport par la membrane vitelline du placenta.

STATEMENT OF THE PROBLEM

The accidental release of methyl isocyanate (MIC) in Bhopal, India, in 1984 resulted in several thousand deaths and chronic toxicity including reproductive toxicity. The very high chemical reactivity of MIC suggested that some of its effects might be caused by its metabolites. Two groups of metabolites of MIC have been identified. These are a reversible glutathione conjugate of MIC, S-(N-methylcarbamoyl)glutathione (SMG) and the methylamines. Methylamines (mono-, di- and trimethylamine) are also endogenous products and their concentration in the body is known to increase in certain disease states such as kidney and liver disease and after ingestion of foods rich in TMA or choline.

The purpose of this research project was to examine the foetal toxicity of TMA and SMG, both for the reasons of their relevance to the toxicity observed in Bhopal as well as possible implications in reproductive abnormalities of certain diseases.

Initial studies revealed that these agents indeed produced foetal toxicity. The main focus of this study was therefore to examine the underlying mechanisms of this toxicity, using mouse embryo cultures and isolated organs.

CLAIMS TO ORIGINALITY

All the experiments contained in this thesis were planned and conducted by the author. To the best of my knowledge, the following are original findings of this study.

1. Methyl isocyanate is directly toxic to embryos.
2. Trimethylamine is selectively foetotoxic *in vivo.*
3. Monomethylamine, dimethylamine and trimethylamine are toxic to embryos *in vitro.*
4. Trimethylamine reduces embryonic growth by an inhibition of receptor-mediated uptake mechanisms of the yolk sac placenta.
5. S-(N-methylcarbamoyl)glutathione (SMG) is toxic to embryos and reduces embryonic growth by an inhibition of uptake mechanisms of the yolk sac placenta.

These findings are contained in the following publications:

1. **Guest, I.** and Varma, D.R. (1990). Developmental toxicity of methylamines in mice. *J Toxicol Environ Health*, **32**:319-330.
2. **Guest, I.**, Baillie, T.A. and Varma, D.R. (1992). Toxicity of the methyl isocyanate metabolite, S-(N-methylcarbamoyl)glutathione on mouse embryos in culture. *Teratology*, **46**:61-67.
3. **Guest, I.** and Varma, D.R. (1992). Selective growth inhibition of the male progeny of mice treated with trimethylamine during pregnancy. *Can J Physiol Pharmacol*, (in press).
4. **Guest, I.**, Cyr, D.G. and Varma, D.R. (1993). Mechanism of trimethylamine-induced inhibition of macromolecular synthesis by mouse embryos in culture (*Can J Physiol Pharmacol*, submitted).
5. **Guest, I.** and Varma, D.R. (1993). Effects of the methyl isocyanate metabolite, S-(N-methylcarbamoyl)glutathione, on mouse yolk sac functions (*Food Chem Toxicol*, submitted).

CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

In the past 30-40 years there have been a number of serious contaminations of the environment as a result of accidental or intentional release of chemicals into the air, water or soil. Some of the most spectacular are oil spills, such as those from the ships *Torrey Canyon* in 1967 (115,000 tons), the *Amoco Cadiz* in 1978 (220,000 tons), the *Exxon Valdez* in 1989 (33,000 tons), the *Brear* in 1993 (64,000 tons) and the 1991 Iraq-Kuwait war, in which an estimated 67 million tons of oil were released into the Arabian Gulf or burned (Perry, 1980; Ollis, 1992; Christie, 1993). These events have considerable effects on ecosystems, especially the benthic community, and kill hundreds or thousands of birds, fish and mammals. Despite the profound immediate effects on wildlife however, evidence collected 4 months after the Gulf war suggest that long-term consequences may be minor (Readman *et al.*, 1992). The effects on human health have yet to be determined.

Accidents in which industrial chemicals contaminate food stuffs have a more direct and lasting effect on humans, causing mortality or morbidity. Some notable examples are the mercury contamination of fish in Minamata and Niigata, Japan during 1953-1960 resulting in 111 cases of serious neurological toxicity (Nomura, 1968); the Yusho oil syndrome in Japan in 1968 and in Taiwan in 1979, in which cooking oil was contaminated with polychlorinated biphenyls (PCBs), resulting in 1,200 cases with 22 deaths and 1800 cases, respectively (Kuratsune, 1976; Hsu *et al.*, 1984); the consumption of methylmercury-treated wheat and barley seeds in Iraq in 1972, resulting in 6,530 cases with 459 fatalities (Bakir *et al.*, 1973) and the Spanish toxic oil syndrome in 1981, when aniline-denatured rapeseed oil was sold as cooking oil, in which 20,000

cases were recorded, with 315 deaths (Tabuenca, 1981; Kilbourne *et al.*, 1983).

In another category, there may be release of toxic chemicals into the atmosphere, to be dispersed and distributed to surrounding areas due to climatic factors. Three recent examples are the release of trichlorophenol contaminated with dioxin at Seveso, Italy in 1976 (Pocchiari *et al.*, 1983), the leak of methyl isocyanate gas in Bhopal, India in 1984 (Varadarajan *et al.*, 1985) and the meltdown of the nuclear reactor in Chernobyl, Russia in 1986, which released mainly ^{131}I and lesser amounts of short-lived iodine isotopes (Kazakov *et al.*, 1992). In the Seveso incident no deaths could be attributed directly to the release but because of the persistence of dioxins, extensive decontamination of the affected land and vegetation had to be undertaken (Hay, 1977). The Bhopal accident is probably the world's worst industrial accident in terms of mortality, with several thousand deaths and considerable morbidity many years after the accident. The official death toll from the Chernobyl reactor explosion is 31 but the long-term effects may well prove to be substantial (Dickman, 1991). For example, thyroid cancer in children living in proximity to Chernobyl appears to be occurring at greatly increased rates (Baverstock *et al.*, 1992).

Paralleling the rise in environmental spills and illness resulting from occupational exposure to toxic chemicals, strict workplace and environmental regulations governing permissible exposure limits have been formulated. With this regulation has come the necessity for animal testing of chemicals for potential human toxicity. These animal tests are initially used to establish safe exposure limits but they are also the usual first step in trying to understand the mechanisms of toxicity involved.

It is a consequence of industrial exposure in general and tragic industrial accidents

in particular that mechanisms of toxicity of several chemicals have been elicited. Prior to the Bhopal accident the limited exposure of humans to methyl isocyanate did not warrant, or there were no regulations requiring, extensive animal testing. At the time of the Bhopal accident, one study on the toxicity of MIC had been published in German (Kimmerle and Eben, 1964). This publication noted the direct irritant effect of MIC on the lungs, skin and eyes, evident at very low (several ppm) concentrations and that MIC caused pulmonary edema. There were also several publications on the toxicity of other isocyanates, chiefly diisocyanate, encountered as a result of occupational exposure. These compounds have a toxicity profile very similar to MIC and since they are still widely used, a brief discussion of isocyanate chemistry and toxicology will be presented. Of particular relevance is the long-term toxicity noted in humans exposed to the isocyanates.

1.1 The Bhopal accident and isocyanate toxicity

1.1.1 Outline of the Bhopal accident

Methyl isocyanate (MIC) is an intermediate used in the plastics and pesticide industries. The Union Carbide pesticide plant located in Bhopal, India, began operations in 1968 and started the production of MIC, used in the manufacture of the carbamate pesticide carbaryl, in 1979. Late in the evening of December 2, 1984, a leak of MIC occurred during maintenance procedures. Approximately 40 tons of MIC escaped under pressure through a 33 metre high vent pipe. Once dispersed and reduced to atmospheric pressure, MIC (vapour density 1.97 relative to air) descended onto the surrounding, heavily populated area. Within 24 hours of the release over 1,000 people had died and 40-50,000 required medical attention. The final death toll probably

exceeded 3,800 (Lepkowski, 1992). The predominant symptoms in survivors were pulmonary dysfunction, skin and eye irritation, vomiting, nausea, unconsciousness and fatigue (Andersson *et al.*, 1984; Kamat *et al.*, 1985; Misra *et al.*, 1987), in accordance with exposure of tissues to a highly reactive chemical in gaseous form. No firm figures are available for the level of exposure but estimates of 75-100 ppm over 1-2 hours have been suggested (see Varma, 1986).

1.1.2 Chemistry and toxicology of isocyanates.

Isocyanates, characterized by the highly reactive -N=C=O group, are chemical intermediates in polyurethane foam, plastics, adhesives, coating materials, pesticides and paint manufacturing (Baur, 1990a). The commonly used isocyanates are toluene diisocyanate (TDI), hexylmethylene diisocyanate (HDI) and diphenylmethane-4,4'-diisocyanate (MDI). Isocyanates cause sensory and pulmonary irritation, which corresponds, respectively, to a burning sensation in the eyes, nose and throat and to a potential for lung damage. Both occur at extremely low concentrations. For example, sensory irritation to TDI in mice, evaluated by a 50% drop in the respiratory rate (RD_{50}), occurs at 0.81 ppm for a 10 minute exposure (Sangha and Alarie, 1979).

Isocyanates are the most common cause of occupational asthma (Cartier *et al.*, 1989). They have been associated with the development of immediate and late asthma, bronchitis, rhinitis, conjunctivitis, chronic obstructive lung disease, contact sensitivity, dermatitis and, rarely, allergic alveolitis and immunologic haemorrhagic pneumonitis (Peters *et al.*, 1968; Charles *et al.*, 1976; Patterson *et al.*, 1990; Karol, 1991). Asthma is the most common disease and it may persist despite removal of the isocyanate; 14% of asthma cases are IgE-mediated. Although toxic mechanisms and IgG-mediated allergic

alveolitis only occur at concentrations above the threshold limit value (TLV), the TLV (10 ppb) is still frequently exceeded in localized environments of many industries (Baur, 1990a). It has been stated that sensitization can occur at any concentration (Baur, 1990b).

Some of the earliest documented toxicity of isocyanates are reports of firemen exposed to diisocyanate liquid and fumes during a fire at a polyurethane foam manufacturing plant. Respiratory (irritation of eyes and throat, chest tightness, breathlessness), gastrointestinal (nausea, vomiting, diarrhoea) and neurological (ataxia, unconsciousness, confusion, poor memory and difficulty concentrating) symptoms occurred either immediately or within 3 days (Axford *et al.*, 1976; Le Quesne *et al.*, 1976). Although in the majority of cases symptoms disappeared over the next 10 days, in about one third of those exposed, pulmonary and neurological dysfunctions, including increased incidence of respiratory infections, intolerance of smoky environments, poor memory, personality changes and depression, persisted 4 years after the initial exposure. In another follow up of exposure for several hours to unspecified high levels of TDI, there was airway dysfunction (wheezing, exertional dyspnoea) and asthma-like symptoms 7 years later (Luo *et al.*, 1990).

Isocyanates induce sensitization and stimulate production of antibodies to protein-isocyanate conjugates; that is, antibodies are directed against altered portions of homologous carrier protein and not to the isocyanate itself (Grammer *et al.*, 1990). Even so, there is specificity of the antibodies produced to the different isocyanate haptens (Cartier *et al.*, 1989; Cvitanovic *et al.*, 1989). There is evidence in mice that, depending on whether an isocyanate is only a contact allergen or also induces pulmonary sensitization, divergent immunological responses involving different antibodies are

stimulated (Dearman *et al.*, 1992).

1.1.3 Transport of isocyanates

The sensitization induced by the isocyanates attests to the highly reactive nature of the chemical group. However, the puzzling feature of the diisocyanates documented above, and the symptoms observed in Bhopal victims, is that significant long-term morbidity occurred, even after a brief exposure. Bone marrow toxicity, foetal toxicity and induction of specific antibodies noted in survivors are difficult to resolve as consequences of pulmonary pathology alone. However, based on a strictly chemical prediction of a short half-life, estimated to be less than 2 minutes in aqueous media for MIC (Brown *et al.*, 1987), the lung would be expected to be the chief target organ. In order for the concept of systemic toxicity to occur after exposure to MIC, one must envisage either sufficient lung toxicity to induce the systemic effects or a preservation of the MIC moiety in some form that can cross cell membranes, in which reactivity is maintained and by which widespread distribution is achieved. This would be one way to explain multiorgan toxicity from a single (lung) exposure. Biochemical evidence accumulated in the past 30 years and especially in the past 5, indicates that isocyanates and some other compounds can combine reversibly with glutathione in a way to maintain activity, a process which not only extends the half-life but allows for distribution throughout the body via the systemic circulation and subsequent release of the parent compound at sites not exposed in the original insult.

In 1986, the cytotoxicity of allyl and benzyl isothiocyanates was characterized *in vitro* (Bruggeman *et al.*, 1986). Glutathione conjugation of these two isothiocyanates is known to be the most important metabolic pathway and conjugation with glutathione

occurs both spontaneously and enzymatically, catalysed by the glutathione *S*-transferases (Brusewitz *et al.*, 1977; Mennicke *et al.*, 1983). The cytotoxicity of the glutathione and cysteine conjugates can be prevented by excess glutathione, indicating that it is the release of the parent isothiocyanate that initiates the toxicity. Conjugation was completely reversible and Bruggeman concluded that these conjugates could act to transport the isothiocyanates to distant sites, where they would be released. In other words, conjugation with thiols extended the half-life and toxicity of the isothiocyanates. A glutathione conjugate of MIC, S-(N-methylcarbamoyl)glutathione (SMG), was isolated in 1990 from the bile of rats administered MIC (Pearson *et al.*, 1990). The role of glutathione conjugation in toxicity and a detailed discussion of SMG occurs in a separate section below.

1.2 METHYL ISOCYANATE (MIC)

1.2.1 Toxicity

1.2.1.1 Pulmonary

Autopsies performed on victims of the Bhopal accident established that the predominant cause of death was cardiac arrest following lung failure due to severe pulmonary damage, the result of pulmonary edema and symptoms resembling acute adult respiratory distress syndrome (Salmon *et al.*, 1985; Kamat *et al.*, 1985; Marwick, 1985). Lungs of victims were commonly 2-3 times heavier than normal and revealed edema, substantial destruction and necrosis of the alveolar wall, desquamative and ulcerative bronchiolitis and infiltration by macrophages (Marwick, 1985).

Examination of 500 exposed people within 3 days of the accident suggested

symptoms of interstitial and/or alveolar edema in 40% and destructive lesions such as cavitation, pneumothorax or emphysema in 8% (Sharma and Gaur, 1987). A retrospective study of 978 patients admitted to local hospitals revealed a mortality rate of 7.14% within 72 hours of admission (Misra *et al.*, 1987). Predominant symptoms in survivors were breathlessness and cough in 95%, irritation and choking in the throat in 46%, pain in the chest (25%), nausea and vomiting (52%), irritation of the eyes (86%) and extreme weakness (25%). In another study of 82 patients within 2 months of the accident, 55% demonstrated inability to maintain normal maximal-minimal ventilation (Kamat *et al.*, 1985). In 95% of these patients, chest radiographs showed extensive changes indicative of interstitial depositions.

In a survey of 282 victims living within a 2 km radius of the MIC plant, more than half demonstrated pulmonary (chest pain, cough, dyspnea), eye (burning watering, aberrant vision), gynaecological and neurological symptoms persisting 100 days after MIC exposure. Only 50% of 82 patients studied three and six months later showed signs of improved lung function as measured by spirometry (Kamat *et al.*, 1987). These same patients studied at 18 months demonstrated persistence of airflow volume reduction and alveolitis (Patel *et al.*, 1987). In another follow-up study, restrictive lung function changes and impaired oxygen exchange in MIC-exposed people had mostly resolved in large airways 2 years later but alveolitis and flow dysfunctions persisted in small airways (Kamat *et al.*, 1992).

Both the acute and the chronic findings have been confirmed and extended in animal models by various investigators. Deaths in rats or mice exposed to MIC vapour usually occurred within 1 week of acute (1-3 hours) inhalation of 10-30 ppm, and was preceded by respiratory distress characteristic of airway obstruction (Alarie *et al.*, 1987;

Bucher *et al.*, 1987b). As with other isocyanates, MIC is a potent sensory and pulmonary irritant in rats and mice (Nemery *et al.*, 1985a). The RD_{50} could be elicited in mice by 1.3 and 1.9 ppm MIC affecting, respectively, sensory and pulmonary mechanisms (Ferguson *et al.*, 1986). Effects were seen at concentrations as low as 0.4 ppm after 90 min of exposure. Low concentrations of MIC, on the order of several ppm, led to proximal airway damage: necrosis and loss of epithelial cells in the trachea, bronchi and bronchioles (Nemery *et al.*, 1985a; Fowler and Dodd, 1987). Small airways were occasionally completely occluded by sloughed epithelial cells, fibrinous and mucus plugs and by inflammation (Gupta *et al.*, 1986; Fowler and Dodd, 1987; Dodd *et al.*, 1987). One week after an acute exposure, inflammation had mostly subsided and sheets of regenerating epithelial cells could be observed (Brody and Hill, 1986). Fibrogenesis accompanied the regeneration, with connective tissue protruding into and disturbing bronchiolar walls (Gupta *et al.*, 1986; Brody and Hill, 1986). Epithelial growth over this fibrosis contributed to the airway obstruction.

Blockade of lower airways by exfoliated cells and mucous that was found in rats immediately after exposure to MIC at 30 ppm for two hours persisted at three months (Bucher *et al.*, 1987a; Boorman *et al.*, 1987). At this time point, nasal mucosa had completely recovered (Uraih *et al.*, 1987). Intraluminal fibrosis, bronchitis and mucous plugs contributed to obstructive lung disease.

Evidence of bronchiolitis and pneumonitis, healing and repair and persistent effects one year after a single acute exposure were found in guinea pigs and rats inhaling MIC (Bucher *et al.*, 1989; Ferguson and Alarie, 1991). There was complete recovery in guinea pigs within 3 weeks after 3 hour exposures to 6 and 13 ppm, but exposure to 19 or 37 ppm led to permanent damage. There appeared to be mechanical limitations to

inhalation and airflow limitations to exhalation. There was increased fibrous tissue in the bronchi and bronchioles and destruction of the alveolar walls and increased septal thickness in the alveoli. Intraluminal fibrosis and subepithelial connective tissue proliferation into the airway lumen was evident in the bronchioles of rats 1 and 2 years after similar acute exposures (Bucher *et al.*, 1989).

1.2.1.2 Ophthalmic

The clinical picture of eye damage immediately after the accident included persistent watering, lid edema, photophobia and ulceration of the corneal epithelium (Andersson *et al.*, 1984). Damage to the cornea was most severe at intermediate levels of exposure in humans (Salmon *et al.*, 1985) and animals (Gupta *et al.*, 1987). High concentrations that led to respiratory distress and death induced copious lacrimation and lid closure which probably protected the eye surface. Even lower concentrations of MIC as conjectured to exist at Bhopal induced these protective mechanisms (and other avoidance behaviours) in humans, which, in combination with hydrolysis of MIC in the tears, is believed to be responsible for the relative lack of permanent eye damage (Andersson *et al.*, 1985).

In follow up studies of unexposed and exposed cohorts 3 years after the accident, exposed residents of Bhopal had an excess of eye irritation, eyelid infection, cataracts and a decrease in visual acuity (Andersson *et al.*, 1986, 1990). Survivors showed full resolution of corneal erosion but demonstrated an increased risk for development of eye infection and irritant symptoms.

1.2.1.3 Neurological

The brains of a number of Bhopal victims were noted to be oedematous and red in colour (ICMR, 1986). There were foci of haemorrhage in the white matter and in some cases intraventricular and intracerebral haemorrhage.

A number of neurological symptoms were noted in survivors at Bhopal. The predominant ones were depression, muscular weakness, unconsciousness, anxiety, tremours and parasthesia (Misra *et al.*, 1987; Kamat *et al.*, 1985). Depression, anxiety, poor concentration and personality dysfunction gradually resolved with time but significant depression persisted 2 years after the accident (Kamat *et al.*, 1992). These are consistent with psychoses noted in other populations that have experienced disasters. Of those exposed to a natural disaster, 30-59% may suffer traumatic neuroses (Raphael and Middleton, 1988).

1.2.1.4 Cellular and Immunological

MIC is an extremely potent myelotoxin, inducing inhibition of erythroid precursors, pluripotent stem cells and granulocyte-macrophage progenitors after inhalation of 1 and 3 ppm in mice. Recovery of this inhibition occurred within 3 weeks after 1 ppm but not after 3 ppm and no other pathological changes were noted (Hong *et al.*, 1987). Cell cycling in bone marrow, alveolar cells and peripheral T lymphocytes was severely inhibited at low (6-15 ppm) concentrations (Conner *et al.*, 1987; Shelby *et al.*, 1987). Persistent dioestrus was induced by 9 ppm MIC when female mice were exposed for three hours (Varma *et al.*, 1987). MIC has also been shown to be toxic in whole brain cell cultures, causing necrosis, although recovery did occur at sub-lethal (1-2 μM) concentrations (Anderson *et al.*, 1990), and in muscle cell cultures, inhibiting

differentiation and formation of muscle fibres (Anderson *et al.*, 1988).

MIC (probably as an albumin hapten) can induce a specific antibody response but the increased levels observed in humans exposed in Bhopal and in experimental animals are transient and not high enough to suggest any detrimental effect on health (Karol *et al.*, 1987). Immunoglobulins of the IgG, IgM and IgE classes were increased but there was no evidence of compromised humoral immunity (Saxena *et al.*, 1991). Cellular immunity was slightly depressed but resistance of mice to bacterial, viral or tumour cells was not affected (Tucker *et al.*, 1987).

1.2.1.5 Reproductive

Epidemiological and experimental evidence collected after the accident strongly suggest that MIC is a reproductive toxin. A survey of 3,270 Bhopal families taken in 1986 (with a follow up in 1990) revealed 865 women who were in various stages of pregnancy at the time of exposure. These women demonstrated gynaecological complications and postnatal effects consistent with systemic toxicity. Of the 865 pregnancies, 379 (44%) did not result in a live birth (Varma, 1987, 1991). Although there are no published figures, estimates put the rate of unsuccessful pregnancies in the Bhopal population prior to the accident at 14-15% (see ICMR, 1986). The neonatal death rate in the exposed population was 15%, in comparison to 2-3% in Bhopal in the previous 2 years. There were other gynaecological abnormalities, including leucorrhea, suppression of lactation and increased incidence of pelvic inflammatory disease (see Varma, 1986).

Reproductive toxicity after MIC inhalation has also been reported in mice and rats. Exposure to 9 or 15 ppm MIC on day 8 of gestation led to resorption of > 80% of implants; three quarters of these mice lost all their implants (Varma *et al.*, 1987). Similar

data were observed in rats. Lower concentrations resulted in significant decreases in foetal and placental weights and in foetal skeletal size. Foetal toxicity did not depend upon maternal pulmonary irritation, as increased resorptions and decreased foetal and placental weights were evident in rats given MIC intraperitoneally (Varma, 1987). Exposure of mice to 1 or 3 ppm MIC on days 14-17 of gestation led to a reduction in litter size and in neonatal survival (Schwetz *et al.*, 1986).

MIC has been shown to be toxic to developing foetuses both *in vivo* and *in vitro* (Varma *et al.*, 1990). When mouse embryos were removed from dams following a 2 hour exposure to 30 ppm MIC and cultured for 48 hours, the embryos from treated dams were significantly reduced in size compared to controls. A similar result was obtained when rat embryos were removed from unexposed dams and then exposed to MIC vapour *in vitro*; exposed embryos demonstrated reduced size and protein content in comparison to control embryos (Varma *et al.*, 1990). MIC was monitored in the culture bottles and found to disappear within minutes of instillation. Formation of toxic metabolites from the reaction between MIC and components of the culture media is a possible explanation for the observed embryotoxicity in the absence of MIC.

S-(N-methylcarbamoyl)glutathione (SMG), the reversible conjugate between MIC and glutathione, has been shown to be toxic to developing foetuses *in vitro*. SMG reduced growth of mouse embryos in a dose-dependent manner and inhibited DNA synthesis without causing significant mortality (Guest *et al.*, 1992). The possibility is thus established that SMG may have contributed to the reproductive toxicity of MIC observed in Bhopal women after the accident.

1.2.1.6 Genetic

Both positive and negative results have been reported on genotoxicity of MIC. Although Conner *et al.* (1987) could not find any chromosomal damage in mouse tissue *in vivo* or *in vitro*, others have documented chromosomal aberrations and increased rates of sister chromatid exchanges, both in animals and in Bhopal victims (Shelby *et al.*, 1987; Goswami, 1986; Goswami *et al.*, 1990), although no clear dose-response relationship could be found (Ghosh *et al.*, 1990).

MIC has been reported to be mutagenic in modified mammalian and bacterial cell cultures (Caspary and Myhr, 1986; Meshram and Rao, 1988). Tests were modified by being conducted at 10 or 25°C in order to reduce the amount of MIC that was hydrolysed and so increase the amount reaching the cells. However, results were negative when the incubation temperature was 37°C. MIC preferentially reacts with the amine groups of deoxycytidine to produce carbamylated DNA but these lesions can be removed by excision repair (Tamura *et al.*, 1992). Thus the general consensus is that MIC can alter chromosomal structure but is not able to induce gene mutations.

1.2.1.7 Biochemical and systemic

Exposure to MIC has led to the appearance of shock-like symptoms. Loss of fluid (Kolb *et al.*, 1987) and increase in the haematocrit (Troup *et al.*, 1987; Jeevarathinam *et al.*, 1988) has been recorded in rats and guinea pigs inhaling MIC and in rabbits administered MIC subcutaneously. In rabbits, the 0.5 and 1 LD_{50} induced an almost 50% drop in blood pressure, stemming from decreased cardiac output due to a loss of vascular fluid. The resulting hypovolaemic hypotension led to stagnant hypoxia and lactic acidosis. If MIC was administered by inhalation, there was an increase in blood pressure

and a resulting bradycardia due to stimulation of respiratory reflexes by MIC (Jeevarathinam *et al.*, 1988). Hyperglycaemia, lactic acidosis, increased plasma urea and severe hypothermia has been recorded in rats dosed for 30 minutes with MIC at 0.5 or 1 LD_{50}, whether the route was inhalational or subcutaneous (Jeevaratnam *et al.*, 1990).

MIC has been shown to inhibit erythrocyte membrane-bound adenosine triphosphatase (ATPase) both *in vivo* and *in vitro* (Agrawal *et al.*, 1990; Jeevaratnam and Vaidyanathan, 1992b). It should be pointed out, however, that the dose required to significantly affect ATPase *in vivo* was 0.5 or 1 LD_{50} and *in vitro*, a single acute exposure (1300 ppm) was much less effective than repeated low (142 ppm) exposures over 10-15 days (Agarwal *et al.*, 1990). MIC has also been shown to reduce ATP stores *in vivo*. Rats were exposed to 25 ppm MIC for 1 hour. A significant reduction was recorded, even 5 and 7.5 months later (Sarma *et al.*, 1990). The authors suggest that this is a possible basis for the noted muscle weakness in survivors of the Bhopal accident.

MIC impairs cellular respiration, inhibiting electron transport at complex 1 in mitochondria and acting as a weak uncoupler of energy transduction (Jeevaratnam *et al.*, 1992). Thus in rats *in vivo* MIC inhibits oxidation of NADH and NAD-linked substrates, resulting in histotoxic hypoxia. Although stagnant hypoxia is believed to be the cause of the lactic acidosis, concurrent development and contribution of histotoxic hypoxia to the pathological changes cannot be ruled out (Jeevarathinam *et al.*, 1988).

A reduction in serum glutathione levels was noted in survivors of the Bhopal accident (Srivastava *et al.*, 1988) and a similar finding has been observed in animals. Rats exposed to a single high (1420 ppm, 8 minutes) concentration or repeated low (355 ppm, 8 minutes) concentrations of MIC demonstrated significant decreases in lung microsomal glutathione content up to three weeks after exposure (Mishra *et al.*, 1991).

Cytochrome P-450 enzyme (aniline hydroxylase and aminopyrene demethylase) activity was also decreased and glutathione-S-transferase activity significantly increased.

1.2.2 Carbamylating activity of MIC

MIC and other cyanates are known to carbamylate protein, including haemoglobin and DNA (Segal *et al.*, 1989; Bhattacharya *et al.*, 1988). MIC is an effective antisickling agent *in vitro*, combining with the α-amino groups of haemoglobin S, conferring increased oxygen affinity and preventing aggregation (Lee, 1976). Free sulphydryl groups were not targeted by MIC and the reaction was irreversible. Although rats and guinea pigs exposed to MIC at several hundred ppm had altered haematological values indicative of generalized hypoxic injury (Troup *et al.*, 1987), no direct effects on haemoglobin could be found (Maginniss *et al.*, 1987). In both rats and guinea pigs *in vivo*, MIC can carbamylate haemoglobin, but only at doses in excess of the LD_{50} and then, only about 2% of haemoglobin was carbamylated (Ramachandran *et al.*, 1988). Thus the possibility that carbamylation of haemoglobin led to the marked tissue hypoxia in Bhopal victims is unlikely (Jeevaratnam and Vaidyanathan, 1992a).

1.2.3 Cyanide controversy

In the hours and days immediately following the release of MIC in Bhopal, there was a great deal of confusion. Doctors were overwhelmed by the number of people seeking treatment and had no idea which chemical was involved nor what antidote to use. Once MIC was suspected, officials from Union Carbide stated that MIC was broken down rapidly and would not result in any long term effects (see Varma, 1986). With the confusion, hysteria, observation of bright red blood in victims and anecdotal reports that

sodium thiosulphate was an effective treatment, it is easy to understand how cyanide became the suspected causative agent.

As pointed out by Salmon (Salmon, 1986), there has not been any data indicating the presence of cyanide as a result of exposure at Bhopal. Even so, increased thiocyanate excretion after sodium thiosulphate therapy and increased baseline levels of thiocyanate in Bhopal populations compared to unexposed controls have been cited as evidence of cyanide involvement (ICMR, 1986). Perhaps more truthfully, it has been said by several Indian officials that the controversy over cyanide is more a sociopolitical than a scientific one (see Lepkowski, 1985). According to at least one community worker, the important issue was to show that the lung damage was not due to endemic disease such as tuberculosis (which, if recognized, would absolve Union Carbide of responsibility) but was a result of exposure to MIC. The data of increased thiocyanate excretion would indicate a specific therapy to a specific toxin, cyanide, and would provide legal evidence in the case against Union Carbide.

The evidence disputing cyanide involvement is compelling. Cyanide has a vapour density of 0.96 (MIC 1.96) so if released high above Bhopal under pressure, would be less likely than MIC to descend to the ground below, at least in proximity to the plant. It has been shown in rats inhaling 60 ppm MIC for 2 hours that blood cyanide levels were no different than in control rats (Jameson *et al.*, 1986). Sodium thiosulphate does not protect against the acute or sub-acute effects of MIC in rats, nor does it prevent mortality (Nemery *et al.*, 1985b; Jameson *et al.*, 1986). In another study in which rats were exposed to 65 ppm MIC for 2 hours, there was reduced thiocyanate excretion compared to unexposed rats (Salmon *et al.*, 1985).

The appearance of bright red blood in victims was cited as evidence of cyanide poisoning, based on the theory that cyanide is preventing tissue uptake of oxygen. The haemoglobin remains oxyhaemoglobin and so bright red. However, blood in MIC-exposed rats was specifically monitored for oxygen levels, which were found to be decreased (Nemery *et al.*, 1985b). Furthermore, in a spectroscopic study of blood to which MIC had been added, the absorption peak of haemoglobin remained unaffected, and the only detectable change could be duplicated by the addition of methylamine, a degradation product of MIC in aqueous media (Salmon, 1986). Thus, although cyanide can be produced by the degradation of MIC, it would appear to be unlikely to play a major role in the aetiology of the observed clinical sequelae after the Bhopal accident.

1.2.4 Degradation and metabolic products of MIC

There was evidence of high temperatures at the MIC storage tank involved in the leak at Bhopal, but based on current knowledge, the clinical data collected after the accident are consistent with MIC being the toxic gas. Thus degradation products can include pyrolysis products, such as cyanide, but are predominantly those produced upon reaction of MIC with water, with other degradation products or with biological tissue. MIC can polymerize or more rarely form trimethyl or dimethyl isocyanates. Reaction of MIC with water yields a multitude of products, including monomethylamine, dimethylamine, trimethylamine, dimethylurea, trimethylurea, dimethyl isocyanate, trimethyl isocyanate, trimethylbiuret and carbon dioxide. Reactions between these products can produce urethane, amides and carbamates (Andersson *et al.*, 1985; Lowe, 1970).

The chemical reactions outlining the production of methylamines from MIC are given below:

$$MIC + H_2O \rightarrow \text{Monomethylamine} + CO_2$$

$$4\ MIC + H_2O \rightarrow \text{Dimethyl isocyanate} + \text{Dimethylamine} + CO_2$$

$$7\ MIC + H_2O \rightarrow 2\ \text{Dimethyl isocyanate} + \text{Trimethylamine} + CO_2$$

The carbamylated products derived from the reaction between MIC and proteins can degrade to amines and carbon dioxide (Lowe, 1970). S-(N-methylcarbamoyl)-glutathione (SMG), formed when MIC is exposed to tissue, can release free MIC (Pearson *et al.*, 1990).

1.2.5 Rationale for studying methylamines and MIC glutathione conjugates

Although MIC can be considered to be a reproductive toxin, its highly reactive nature and short half-life suggested that metabolic products contributed to or indeed were responsible for the chronic sequelae. For example, the induction of immunological responses, bone marrow depression and cytogenetic damage would seem to be unlikely events following an acute exposure of a chemical as reactive as MIC. Several of the many degradation products of MIC have been shown to be toxic either in humans or animals. I chose to investigate methylamines and S-(N-methylcarbamoyl)glutathione (SMG) for a number of reasons. First, since human exposure occurred with MIC in the vapour phase, products with potential roles in reproductive toxicity would have to be formed at the sites of exposure under physiological conditions. Human lung alveolar fluid contains high concentrations of glutathione (Cantin *et al.*, 1987), so formation of SMG

from inhaled MIC could occur in the lungs. If MIC crossed cell membranes, either in its native form or as the glutathione conjugate, further interaction with glutathione would be possible, since GSH is a common cellular constituent, with concentrations ranging up to 10 mM (Meister, 1988). Alveolar fluid and the mucus lining of the respiratory tract could certainly provide the aqueous environment necessary for the production of the methylamines.

Second, monomethylamine and dimethylamine are endogenous compounds and trimethylamine is supplied exogenously in dietary ingredients. Humans are therefore commonly exposed to these aliphatic amines on a regular basis.

Third, serum concentrations of methylamines increase in certain diseases. These include liver and kidney disease, such as chronic renal failure (de La Huerga and Popper, 1951; Simenhoff *et al.*, 1963). There is also an inherited disorder, trimethylaminuria, in which patients lack the ability to convert TMA to its oxide, thus demonstrating increased urinary excretion of TMA (Humbert *et al.*, 1970). Ingestion of foods rich in TMA or precursors also increases serum concentration of TMA (Zeisel *et al.*, 1983; Zeisel and DaCosta, 1986). Thus there are clinical conditions in which serum methylamines are increased. The fact that exposure to the methylamines is not limited to prior exposure to MIC implies that research on these compounds may yield results applicable to clinical conditions. A concise background on the methylamines and the glutathione conjugate is presented below.

1.3 Methylamines

The methylamines are simple aliphatic compounds which may be regarded as ammonia in which one or more hydrogen atoms are replaced by methyl groups.

Monomethylamine (MMA), dimethylamine (DMA) and trimethylamine (TMA) are naturally occurring substances in biological tissues and food. Tetramethylamine is not a common ingredient in food in its parent form but it does occur in choline esters and alcohols; discussion will be limited to relevant pharmacological and toxicological features.

1.3.1 Source

Figure 1 gives an outline of the metabolic pathway of methylamines in humans. Although TMA is supplied exogenously, it is of sufficiently widespread occurrence in foodstuffs to allow one the assumption that exposure is common. In fact, humans normally have very low concentrations of all three methylamines, but the levels can fluctuate according to dietary intake. Marine fish contain high concentrations of trimethylamine oxide (TMAO), which is the major end product of nitrogen metabolism in teleosts and elasmobranchs (Parkin and Hultin, 1982). DMA and TMA are released from TMAO both by endogenous fish muscle enzymes and by intestinal bacterial enzymes of humans (Asatoor and Simenhoff, 1965; Lundstrom and Racicot, 1983). Food sources rich in TMA, DMA and MMA include fish, meat, dairy products and spices (Pfundstein *et al.*, 1991). Choline, lecithin and betaine, components of cell membranes and present in eggs, meat, yeast etcetera also yield DMA and TMA upon metabolism by enzymes within the gut flora. Choline is much more efficiently converted to TMA than betaine or lecithin (Lowis *et al.*, 1985). A small amount of DMA is derived from ingested TMA but the predominant source of DMA is endogenous, probably by methylation of MMA (Asatoor and Simenhoff, 1965). MMA is derived from the metabolism of DMA and TMA and from the metabolism of endogenous compounds such as sarcosine, creatine and epinephrine (Schayer *et al.*, 1952; Davis and De Ropp, 1961).

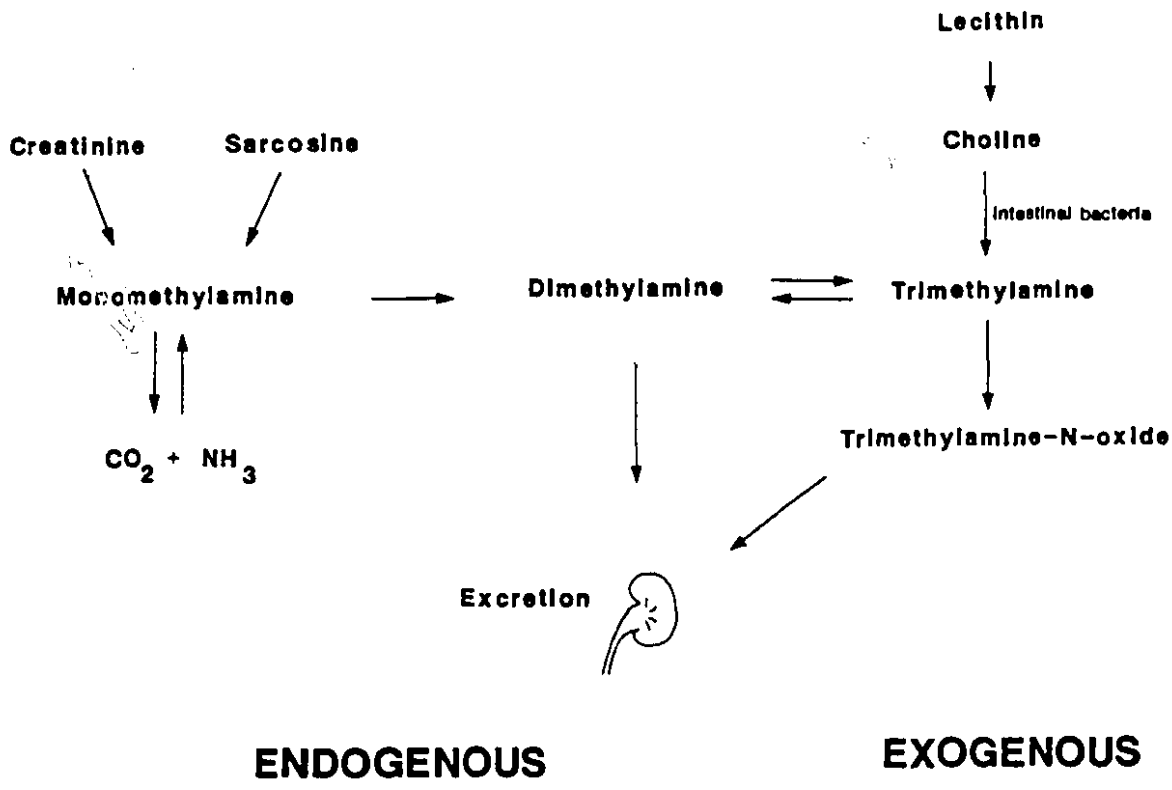

Figure 1. Metabolic pathway of methylamines in humans (adapted from Asatoor and Simenhoff, 1965).

1.3.2 Metabolism

Several different enzymes are responsible for the metabolism of the methylamines. TMA is oxidized to trimethylamine-N-oxide (TMAO) by a hepatic flavin-containing monooxygenase (Baker and Chaykin, 1962; Zeigler and Mitchell, 1972). This enzyme also oxidizes other tertiary and secondary (but not primary) amine drugs, including nicotine, morphine, chlorpromazine and the tricyclic antidepressants (Bickel, 1969). The tertiary amine oxides are reduced back to the tertiary amine, to the secondary amine and to an aldehyde. In the presence of cysteine and iron or haemoglobin, this reduction readily occurs non-enzymatically (Vaisey, 1956) but it is also catalysed by a cytochrome P-450 enzyme (Ackermann *et al.*, 1927; Sugiura *et al.*, 1976). The flavin-dependent enzyme oxidizes secondary amines to hydroxylamines, which are rapidly reduced to the parent amine or to nitrones, which degrade spontaneously (Kadlubar *et al.*, 1973).

Although many tertiary and secondary amine drugs are dealkylated by a cytochrome P-450 monooxygenase (McMahon, 1966), TMA is an exception to this. More than 95% of an ingested dose of TMA is oxidized and excreted within 24 hours (Al-Waiz *et al.*, 1987b). TMA and DMA are not substrates for monoamine oxidase (MAO) A or B. MAO is a cytochrome containing monooxygenase; MAO B is preferentially active on primary and secondary amines containing side chains with 4 or more carbons (Yu, 1989).

MMA is metabolized by two distinct enzymes, plasma monoamine oxidase and semicarbazide-sensitive amine oxidase present in blood vessel smooth muscle cells (McEwan, 1965; Lyles and Singh, 1985). SSAO can also deaminate other aliphatic primary amines containing up to 18 straight chain carbons (Yu, 1990).

1.3.3 Biochemical effects

The methylamines have proven to be useful analytical tools in the examination of many cellular functions and in the understanding of receptor and particle flow across membranes. They have also contributed to our knowledge of enzyme structure and stability and early cellular events during development. It must be noted that most if not all of the biochemical events studied with the aid of the methylamines are conducted with concentrations of 1-50 mM, which are in most cases non-physiological concentrations.

The methylamines belong to a group of compounds known as lysosomotropic agents, that is, substances which are taken-up selectively into lysosomes. Methylamines accumulate in the lysosomes due to their weak base properties; the lysosomal pH is about 4.5-4.8. Methylamines are only permeable in their non-ionized form and once inside a lysosome become ionized and therefore trapped. This ion-trapping leads to rapid increases in the intralysosomal pH of 1-2 pH units which is reversible upon removal of the base (Reijngoud *et al.*, 1976; Ohkuma and Poole, 1978; Poole and Ohkuma, 1981). Cytoplasmic pH is only slightly increased, even at concentrations of 10-20 mM (Tapper and Sundler, 1990). Tetramethylamine is a permanently ionized cation and so is not lysosomotropic. The pharmacological, toxicological and pathological profile of lysosomotropic agents is intimately related to their localization in these intracellular organelles (De Duve *et al.*, 1974).

Lysosomes contain proteolytic enzymes optimally active at pH 4-6 and inactive at pH 7-8. Lysosomotropic agents such as the ionophores monensin and nigericin, which equalize the lysosomal pH with the cytoplasmic pH, and the drug chloroquine, inhibit lysosomal activity at μM concentrations; methylamines are much less potent. A 50%

inhibition of lysosomal proteolysis in isolated hepatocytes requires 10 mM methylamine: monomethylamine, dimethylamine and trimethylamine are equipotent (Seglen and Gordon, 1980). Complete cessation of the lysosomal pathway of protein degradation requires 20-40 mM. Lysosomes are essential organelles in the provision of amino acids for protein synthesis, so that inhibition of lysosomal degradation inhibits not only proteolysis but also protein synthesis, to the same extent as protein degradation (Seglen and Gordon, 1980; Livesay *et al.*, 1980).

Methylamines or the chemically and structurally analogous weak base ammonium chloride interfere in membrane flow and as a consequence prevent the recycling of various receptors, including α_2-macroglobulin (Van Leuven *et al.*, 1980), insulin (King *et al.*, 1981), EGF (Matrisian *et al.*, 1987), LDL (Grant *et al.*, 1990) and mannose receptors (Tietze *et al.*, 1982).

The rise in lysosomal pH induced by methylamines, either alone or in combination with effects on membrane flow, has several consequences. Several hormones or growth factors that bind to their receptors at the cell surface require processing in a low pH environment for uncoupling and subsequent signalling. Concentrations of methylamines that do not interfere in binding or uptake can inhibit dissociation of EGF from its receptor by 95% (King *et al.*, 1981; Widelitz *et al.*, 1984). Inhibition of cell entry or infection by several parasites, viruses or their toxins by methylamines is due in part to the induced rise in lysosomal or endosomal pH and in part to interruption of normal endosome cycling. Diphtheria and clostridial neurotoxin entry and Semlicki Forest virus and *Leishmania* species' infections can be inhibited by methylamines (Draper and Simon, 1980; Simpson, 1983; Helenius *et al.*, 1982; Hunter and Coombs, 1991). The lysosomotropic property has been exploited in the construction of detergents. When a

methylamine is attached to a hydrophobic side chain, with an optimal length of 10-12 carbons, the resulting compound crosses cell membranes and accumulates in lysosomes, leading to rupture of the lysosomal membrane and cell death (Firestone *et al.*, 1979).

Various ammonium salts and methylamines at a concentration of 3 mM have been found to inhibit rRNA synthesis in *Xenopus laevis* embryonic cells by inducing a slight (0.1 unit) rise in cytoplasmic pH. Interestingly, ammonia has been found to occur in unfertilized eggs and embryos at an intracellular concentration of 3 mM (Shiokawa *et al.*, 1987). Both MMA and TMA have been shown to block RNA synthesis. The slight rise in pH that they cause inhibits formation of the 40S pre-rRNA, without inducing breakdown or abnormal processing (Shiokawa *et al.*, 1986, 1987), probably by preventing rDNA transcription. The fact that intracellular pH plays a role in regulating protein synthesis in *Xenopus* oocytes (Houle and Wasserman, 1983) suggests that these amines may act as endogenous regulators of gene expression in early development.

Monomethylamine (MMA) has been shown to interact with several structurally related serum proteins. MMA but not secondary or tertiary methylamines can inactivate the complement components C3 and C4. At a concentration of 2 mM, MMA completely and irreversibly inhibits the haemolytic activity of C4 (Pillemer *et al.*, 1941; Gorski and Howard, 1980). The proteinase inhibitors α_2-macroglobulin and pregnancy zone protein (PZP) are also inactivated by MMA. MMA induces a conformational change which prevents binding of proteinases and so substrate access (Eccleston and Howard, 1985; Christensen *et al.*, 1989).

1.3.4 Methylotrophy

An interesting aspect of methylamine biochemistry and physiology, indicative of the importance of these compounds in primitive life forms, is the reliance some bacteria have on these compounds. Methylotrophy is the obligative or facultative use of compounds with one or more carbon atoms but no carbon-carbon bonds (Colby and Zatman, 1973). This includes all the methylamines as well as other compounds such as formaldehyde, formate and methanol. Obligative methylotrophs rely exclusively on these carbon compounds as the sole source of carbon and energy. Facultative methylotrophs, which possess a complete tricarboxylic acid cycle, can utilise other substrates in the absence of methylamines. Growth of some of these bacteria is exclusively restricted to a single amine, such as TMA (Oren, 1990). A recent extensive monograph documents these fascinating organisms (Anthony, 1982).

Methylotrophy is not restricted to prokaryotes. There are at least three species of mycelial fungi known to be methylotrophic and several hundred strains of yeast which although they cannot use the methylamines, can live off other simple carbon compounds, chiefly methanol (Anthony, 1982). The metabolic pathway in these bacteria is:

$$\text{TMA} \rightarrow \text{DMA} \rightarrow \text{MMA} \rightarrow \text{formaldehyde} + \text{ammonia.}$$

The formaldehyde, as C_1, is assimilated by the ribose phosphate, serine or (if it exists) tricarboxylic acid pathway. Monomethylamine can serve as the C_1 precursor in plants and animals. For example, in tea shoots, methylamine is incorporated into purine nucleotides, amino acids, theobromine and caffeine (Suzuki, 1973) and in animals it is incorporated into DNA, RNA and protein (McNulty *et al.*, 1983).

1.3.5 Pharmacology

The presence of a positively charged nitrogen in the methylamines confers pharmacological activity, although monomethylamine and dimethylamine have no documented pharmacology. Tetramethylamine is the prototypical onium compound active at ganglia. The quaternary nitrogen confers potency but is not essential for activity. In fact, there are secondary (e.g. nornicotine) and tertiary (e.g. nicotine) ganglionic stimulants and the nitrogen atom can be replaced by sulphur (e.g. trimethylsulphonium) and the compound still retain activity (Ing, 1956). The quaternary ammonium moiety is active in ganglionic stimulants and blockers, in cholinergic agonists and in neuromuscular junction agonists and depolarizing agents.

Tetramethylamine does and trimethylamine does not stimulate ganglionic nicotinic receptors (Ing, 1956; Anthoni *et al.*, 1991). The stimulation induced by tetramethylamine differs from nicotine in that stimulation is not followed by a blockade. The tetramethylammonium group is the essential molecular moiety in acetylcholine and muscarine; absence of this group abolishes muscarinic activity (Albert, 1979). Both trimethylamine and tetramethylamine are active but not potent drugs at postganglionic muscarinic receptors. In the guinea pig ileum assay, the EC_{50} values for maximal contraction by carbachol, trimethylamine and tetramethylamine were 0.1, 2000 and 80 μM, respectively (Hayes *et al.*, 1988). Dimethylamine and trimethylamine oxide were inactive. At the ganglia, tetramethylammonium is equipotent to acetylcholine but at the neuromuscular junction acetylcholine is 1,000 times more potent than tetramethylammonium (Albert, 1979).

TMA does have pharmacological activity on nicotinic receptors at the neuromuscular junction. However, the initial stimulation requires 1-8 mM and the

subsequent blockade, due to receptor desensitization, requires 8-32 mM. (Anthoni *et al.*, 1991). Thus trimethyl- and tetramethylamine can be described as having predominantly parasympathomimetic activity.

An extensive structure activity study of sympathomimetic amines was undertaken to examine the effects of aliphatic and aromatic amines (Barger and Dale, 1910). Despite the drawbacks inherent in these whole animal studies, their conclusion that no significant sympathomimetic activity existed in amines of less than 4 carbons remains valid (Blaschko, 1946).

Tetraalkylammonium compounds are known to block cation conductance in a wide variety of K^+ channels, with no or varying degrees of voltage dependence (Tinker *et al.*, 1992). Tetramethylammonium and analogs have recently been shown to block the Ca^{2+}-release channel of sheep heart sarcoplasmic reticulum and to block potassium-induced histamine release in mast cells (Tinker *et al.*, 1992; Nemeth *et al.*, 1990).

Although trimethylamine and tetramethylamine are not themselves used therapeutically, choline and choline esters containing the methylammonium group are still prescribed. Choline and analogs have been studied as replacement therapy in some neurological diseases. Cholinergic deficiencies in Huntington's chorea, tardive dyskinesia, presenile dementia and the Tourette syndrome led to the postulation that choline supplementation might be of clinical use (see Barbeau, 1978). Though some positive results have been reported, recent papers suggest that the issue is controversial. For example, oral choline intake can protect against stimulus-induced loss of striatal membrane phospholipids and can increase intracellular choline. However, acetylcholine synthesis, at least *in vitro*, was not increased by exogenous choline and the therapeutic benefit appears small in clinical studies (Wecker, 1990; Wurtman *et al.*, 1990). The

choline esters acetylcholine, carbachol and bethanechol are used for gastrointestinal and bladder disorders and in ophthalmology (see Taylor, 1990).

The tertiary or quaternary ammonium structure is the basis for various other classes of drugs. The positively charged quarternary nitrogen characterizes cationic detergents and confers antimicrobial activity to various compounds, chiefly betaine esters (Linstedt *et al.*, 1990). The trimethylamine moiety is an active part of carboxyborane and selenium compounds which have antineoplastic activity (Hall *et al.*, 1990; Ip and Ganther, 1988).

1.3.6 Toxicology

In 1947, the American Industrial Hygiene Association issued a relative toxicological scale in order that new chemicals may be classified and handled safely (Dubois and Geiling, 1959). The scale is still widely used today and is reproduced below (the numbers refer to the expected lethal dose in humans):

1.	Extremely toxic	1 mg/kg or less
2.	Highly toxic	1-50 mg/kg
3.	Moderately toxic	50-500 mg/kg
4.	Slightly toxic	0.5-5 g/kg
5.	Practically nontoxic	5-15 g/kg
6.	Relatively harmless	> 15 g/kg

Based on animal studies, methylamines would be rated as moderately toxic. The oral and i.p. LD_{50}'s of TMA in the mouse are 1,680 and 190 mg/kg; the corresponding

values for TMAO are 15,000 and 2,850 mg/kg (Cheymol *et al.*, 1967). Mice administered TMA exhibit muscular twitching, unsteady gait, hypersecretion of the nose and mouth and dyspnoea. Death occurs on the appearance of tonic and clonic convulsions (Cheymol *et al.*, 1967; Anthoni, 1991).

There are a number of suspected uraemic toxins in chronic renal failure, including the methylamines (Bergstrom and Furst, 1978). Serum concentrations of DMA and TMA are consistently raised in uraemia and other kidney diseases and are on average 4-5 times those of control subjects (Simenhoff *et al.*, 1963; Simenhoff, 1975). The intestinal bacterial flora in uraemics is quantitatively greater and qualitatively different from that in normal individuals and its contribution to the raised amine levels in uraemics has been verified by the observation that antibiotic therapy reduces the bacterial flora, decreases serum DMA and TMA concentration and improves clinical symptoms (Simenhoff *et al.*, 1978).

Methylamines are freely diffusible into all body compartments, including the CNS, where they preferentially distribute in the cortical grey areas (Simenhoff, 1975; Moore *et al.*, 1992). There is evidence that TMA may be of greater toxicological relevance than DMA. A good correlation exists between serum concentrations of DMA and TMA and toxic neurological sequelae, but the correlation appears stronger for TMA (Simenhoff *et al.*, 1977) and intraventricular injection of TMA but not of DMA in rats is associated with abnormal behaviour (Simenhoff *et al.*, 1978). There is significant intracellular sequestration of methylamines such that serum levels do not reflect body burden and intracellular concentrations of TMA are greater than DMA (Ihle *et al.*, 1984).

All three methylamines can serve as precursors in the formation of N-dimethyl-nitrosamine (NDMA). NDMA has been documented to methylate nucleic acids both

in vitro and *in vivo* (Magee and Farber, 1961; Magee and Hultin, 1961). NDMA is a potent carcinogen in animals (Magee and Barnes, 1967; Shank, 1975) but there is no conclusive data to suggest that exposure of NDMA in humans is sufficient to induce cancer (Craddock, 1990). Fish (Zeisel and DaCosta, 1986), choline and analogues (Zeisel *et al.*, 1989) and a variety of dietary quaternary amines (Fiddler *et al.*, 1972) have all been shown to act as precursors to NDMA, both *in vitro* and *in vivo*.

1.3.7 Changes in endogenous levels

Consumption of foods rich in methylamines or their precursors (TMAO, choline) results in increased excretion of methylamines and their metabolites (Zeisel and DaCosta, 1986). Saltwater fish, egg yolks, meat and dairy products and some vegetables, especially soybeans, are all high in TMA, TMAO or choline. Up to two thirds of ingested choline is excreted either as TMA or the oxide. More than 90% of ingested DMA and TMA are excreted within 12 and 24 hours, respectively, but their half-lives in the body can be increased by several conditions. Renal disease (chronic renal failure) and hepatic disease results in increased serum DMA and TMA (de la Huerga and Popper, 1951; Marks *et al.*, 1978; Asatoor *et al.*, 1963; Ihle *et al.*, 1984). The inherited disorder trimethylaminuria is characterized by a fishy odour to sweat, breath and urine. Afflicted individuals have a deficiency in converting TMA to the oxide, resulting in excessive excretion of TMA. In most cases methylamine-rich foods exacerbate the condition so that omitting such foods from the diet is effective therapy but in some afflicted individuals, dietary modification cannot control the odour problem (Humbert *et al.*, 1970; Brewster and Schedewie, 1983; Danks *et al.*, 1976).

1.4 Glutathione and toxicity

Xenobiotics are metabolized by different mechanisms in the body which have been conventionally regarded as detoxification. Phase I of this process involves oxidation, reduction, dealkylation etcetera by various enzymes. In phase II xenobiotics, which may be reactive intermediates, are conjugated with glucuronide, glutathione etcetera (Williams, 1959). For many drugs and compounds, glutathione conjugation, the first step in mercapturic acid formation, terminates drug action and increases polarity, making the drug more easily excretable. In the case of prodrugs, metabolism releases the active form and so initiates pharmacological activity. With other compounds metabolism, especially conjugation, can increase toxicity. The glutathione conjugate itself may be the toxic moiety or further metabolism to a reactive intermediate may be necessary before toxicity becomes overt. These toxic species may interact with endogenous proteins to inhibit enzymes, alter structure or induce tumour formation.

1.4.1 Glutathione and detoxication

The conjugation of glutathione with xenobiotic compounds in order to facilitate excretion has been known since the 1960's. Work of Booth, Boyland and Barnes, among others, defined the metabolic route of detoxication by mercapturic acid synthesis, the enzymatic catalysis of glutathione conjugation with electrophilic intermediates and the role of glutathione conjugation in the mercapturic acid pathway (Barnes *et al.*, 1959; Bray *et al.*, 1959; Booth *et al.*, 1960; Boyland *et al.*, 1961).

By 1970 it was well established that glutathione was involved in the conjugation of a variety of xenobiotics which were excreted as mercapturic acids. Cysteine and glutathione were shown to be protective against cellular toxicity of alkylating metabolites

of acetaminophen (Boyland and Chasseaud, 1969; Mitchell *et al.*, 1973). Cyclophosphamide, another alkylating agent, induced teratogenicity which could also be prevented by GSH (Ashby *et al.*, 1976). It was soon realized that many toxic agents depleted cellular GSH and pretreatment with cysteine or glutathione could reduce the toxicity of such agents (see Ketterer, 1986). Many toxins are metabolized to free radical intermediates, including superoxide, hydrogen peroxide and hydroxyl radicals. These radicals deplete glutathione by oxidation and conjugation and by the formation of mixed disulphides. Membrane damage, disrupted calcium homeostasis and induction of proteolytic enzymes may follow, leading to cell death (Bellomo and Orrenius, 1985).

1.4.2 Glutathione and intoxication

In the late 1970's and early 1980's evidence began to accumulate that glutathione played an essential role in the toxicity of some compounds, chiefly haloalkanes and alkenes. The first papers indicated that glutathione conjugation was essential for the mutagenicity of dibromo- or dichloroethane (Rannug *et al.*, 1978; van Bladeren *et al.*, 1980) and in 1983 it was shown that the glutathione conjugate was necessary for DNA adduct formation (Ozawa and Guengerich, 1983). The kidney-specific toxicity of the haloalkenes was shown to be correlated with glutathione conjugation and dependent upon activation by renal enzymes and selective uptake by tubular cells (Dohn *et al.*, 1985; Elfarra *et al.*, 1986). These pathways were confirmed *in vivo* by Guengerich *et al.* (1987).

Glutathione conjugation occurs predominantly in the liver and is catalysed by cytosolic and microsomal glutathione *S*-transferases but highly electrophilic compounds can combine with glutathione non-enzymatically. In some cases, the glutathione

conjugate produced is directly toxic, as with 1,2 dibromoethane and 1,2-dichloroethane (Guengerich *et al.*, 1987). In other cases, processing to a cysteine conjugate and then to a reactive intermediate via the cysteine conjugate ß-lyase pathway is a necessary step in the development of toxicity.

Selective concentration of the glutathione and/or cysteine conjugate by renal tubular cells contributes to the expression of toxicity. In normal detoxication, the cysteine conjugate is acetylated to the corresponding mercapturic acid and excreted. It is not yet clear why cysteine conjugate ß-lyase activation rather than acetylation takes place.

1.4.3 Classification of toxic glutathione conjugates

Four classes of toxic glutathione (GSH) conjugates have been identified, based on chemical similarities of the electrophilic species (Monks *et al.*, 1990; Koob and Dekant, 1991).

The first group are electrophilic sulphur mustards resulting from the reaction between GSH and haloalkanes, such as 1,2-dichloroethane and 1,2-dibromoethane. These compounds can follow two metabolic pathways, cytochrome P-450-dependent oxidation and GSH conjugation (Guengerich *et al.*, 1980). The acetaldehydes resulting from P-450 activation directly bind to proteins (van Bladeren *et al.*, 1980) while the glutathione conjugates can bind to DNA and are responsible for the genotoxicity of these haloalkanes (van Bladeren *et al.*, 1980). Haloalkanes are known to deplete hepatic GSH. Excess N-acetylcysteine can significantly inhibit and depletion of GSH with buthionine sulfoxime can significantly increase the toxicity of 1,2-dichloropropane (Imberti *et al.*, 1990).

The second class contains nephrotoxic haloalkenes. These compounds are conjugated with GSH, converted to the cysteine conjugate and then metabolised to a reactive intermediate by cysteine conjugate ß-lyase. The glutathione conjugate is formed in the liver then transported to the kidney. The liver contains several-fold greater concentrations of GSH *S*-transferases than any other organ and the kidney contains most of the body's gamma-glutamyltransferase and cysteine conjugate ß-lyase (Elfarra and Anders, 1984; Lash *et al.*, 1986). Proximal tubular cells on both the basolateral and tubular side actively concentrate the GSH conjugates by a carrier-mediated process and convert them to the cysteine conjugate and then to a reactive intermediate. Inhibition of gamma-glutamyltranspeptidase by acivicin and of cysteine conjugate ß-lyase by aminooxyacetic acid as well as inhibition of active uptake by probenicid all inhibit the toxicity of these renally-activated glutathione conjugates (Elfarra *et al.*, 1986).

The third group consists of quinones, compounds which readily undergo oxidation-reduction recycling and combine with GSH enzymatically and non-enzymatically, reducing the quinone to a hydroquinone. The GSH-hydroquinone conjugate may then be transported to other sites and cause toxicity due to the ease with which the hydroquinone is oxidized back to the quinone (Monks and Lau, 1990). The toxicity of these conjugates is inhibited by GSH, cysteine and ascorbic acid, by preventing autooxidation of the hydroquinone (Tayama and Nakagawa, 1991). The toxicity is also inhibited by acivicin but not by aminooxyacetic acid, indicating that activation of the cysteine conjugate to a reactive intermediate by cysteine conjugate ß-lyase is not required (Monks and Lau, 1990).

The fourth group results from the reaction of GSH with thiocyanates and some unsaturated compounds. This class is distinguished by the reversible nature of the

conjugate; the parent compound can be released under certain physiological conditions. This class is discussed in detail below.

1.4.4 Reversible glutathione conjugation

Recognition of the role of the isocyanate group in reversible glutathione conjugation preceded that of glutathione. The reversibility of a reaction between an isocyanate and an endogenous compound was first demonstrated *in vitro* in 1973 (Twu and Wold, 1973), although as early as 1964 Stark had demonstrated that potassium cyanate combined reversibly with cysteine (Stark, 1964). The combination of *n*-butylisocyanate and yeast alcohol dehydrogenase was shown to be reversible, with complete restoration of enzyme activity upon release of the isocyanate (Twu and Wold, 1973). Such release occurred at slightly alkaline conditions, at or above pH 7.0. In 1979, cysteine and glutathione conjugates of *n*-butylisocyanate were identified in animals dosed with the pesticide benomyl (Axness *et al.*, 1979). Drugs are also known to release an isocyanate following conjugation with glutathione.

There are now several distinct classes of chemicals which form reversible glutathione conjugates. All of them have one thing in common, a functional ketone group. These classes have been recently reviewed (Monks *et al.*, 1990; Baillie and Slatter, 1991) and are summarized below.

Isothiocyanates and isocyanates. The ability of some compounds to combine with glutathione in such a way as to be releasable in the parent form was first documented with isothiocyanates *in vitro* in 1977 and *in vivo* in 1986 (Brusewitz *et al.*, 1977; Bruggeman *et al.*, 1986). Isothiocyanates are ingredients of vegetables and spices and

this is the principal means of exposure in man, while isocyanates are industrial chemicals used in plastics and pesticide manufacturing, the workplace being the predominant site of exposure. Conjugation with glutathione is a major pathway in the metabolism of allyl and benzyl isothiocyanates, the major isothiocyanates of foods, in animals and humans (Mennicke *et al.*, 1983). Free isothiocyanate can be released in urine from the mercapturic acid derived from the isothiocyanate. In cell cultures, the cysteine and GSH conjugates are cytotoxic and this toxicity can be prevented by excess cysteine or GSH (Bruggeman *et al.*, 1986). It has been shown *in vivo* that the toxicity of α-naphthylisocyanate can be significantly decreased by depletion of GSH (Dahm and Roth, 1991). Formation of a glutathione conjugate of methyl isocyanate was first reported in 1990 (Pearson *et al.*, 1990). This conjugate is discussed in a separate section below.

Formamides, alkyl carbamates and ureas. The common feature of these functional groups, present in drugs and agricultural chemicals, is that they undergo metabolic activation to isocyanates which react with glutathione. N-methylformamide, an antitumour agent and the bronchodilator prodrug bambuterol both form MIC, and the subsequent glutathione conjugate is believed to play an important role in the toxicity of these drugs. The urea derivatives benomyl and caracemide, an antitumour drug, exert their toxicity via formation of GSH and cysteine conjugates with subsequent release of isocyanates (Axness *et al.*, 1979; Newman and Farquhar, 1987). 1,3-*Bis*-(2-chloroethyl)-1-nitrosourea (carmustine), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (lomustine) and the sulfonylurea chlorpropamide form GSH conjugates which probably play a role in the antineoplastic activity as well as the toxicity of these compounds (Gibson and Hickman, 1982; Nagasawa *et al.*, 1989).

α, ß-Unsaturated carbonyl compounds. A few examples exist of the formation of

glutathione conjugates with carbonyl compounds (acrolein, muconaldehyde, furazolidone). Although these reactions are reversible, the release of the parent compound is usually slow and attempts to use the GSH conjugate as a delivery system for the cytotoxic aldehyde have not been very successful (Tillian *et al.*, 1976).

Simple aldehydes. GSH is known to spontaneously react with formaldehyde and acetaldehyde *in vivo*, and these conjugates exist in equilibrium with the parent aldehyde (Naylor *et al.*, 1988). Recent work suggests that GSH may act to transport formaldehyde and act as a cofactor in its oxidation. Since formaldehyde can cross-link DNA there is the potential for toxicity (Baillie and Slatter, 1991).

1.4.5 S-(N-methylcarbamoyl)glutathione (SMG)

S-(N-methylcarbamoyl)glutathione was first documented as a metabolite in mice administered N-methylformamide (NMF), a structural analog of methyl isocyanate. NMF is an antitumour drug that is suspected to revert briefly to MIC during oxidation and conjugation with glutathione (Kestell *et al.*, 1986). Both the GSH conjugate and the mercapturic acid can be easily hydrolysed to the parent thiol (Threadgill *et al.*, 1987). NMF rapidly and significantly depletes GSH in the liver of mice and this depletion and toxicity can be prevented by prior treatment with cysteine or GSH or attenuated by prior depletion of GSH (Pearson *et al.*, 1987a). Pretreatment with GSH also prevents the protein-associated binding of ^{14}C after administration of ^{14}C-NMF (Pearson *et al.*, 1987b). It is now believed to be a common property of alkylformamides that glutathione conjugates mediate their cytotoxicity and antineoplastic activity (Kestell *et al.*, 1987).

In the aftermath of the Bhopal accident it was proposed that MIC might be converted to a transportable form by binding to sulphydryl groups in haemoglobin or glutathione (Brown *et al.*, 1987). Two papers appeared in 1988 indicating that this might

indeed be occurring. Ferguson showed that inhalation of ^{14}C-methyl isocyanate in guinea pigs and mice resulted in rapid and widespread distribution of radioactivity in most organs, including the brain and foetus (Ferguson *et al.*, 1988). Rats administered ^{14}C-MIC by intraperitoneal injection or inhalation demonstrated extensive tissue binding of ^{14}C within 30 minutes of exposure (Bhattacharya *et al.*, 1988). Although the exact chemical nature of the radioactivity binding to the tissues in these experiments was not determined, the data is consistent with MIC being transported as a glutathione conjugate via the systemic circulation. In 1990, SMG was isolated from the bile of rats administered MIC (Pearson *et al.*, 1990). It has since been shown that glutathione conjugation is a major pathway for the metabolism of MIC; 25% of the dose appears as the mercapturate within 24 hours (Slatter *et al.*, 1991).

At physiological pH and temperature *in vitro*, SMG and S-(N-methylcarbamoyl)-cysteine (SMC), the cysteine conjugate of MIC, readily donate their isocyanate to nucleophilic acceptors (Pearson et al., 1990). Isocyanate-glutathione conjugates are suspected to induce toxicity by releasing free isocyanates at cell membranes or in physiological conditions that favour dissociation, such as low GSH concentrations or an alkaline pH (Bruggeman *et al.*, 1988; Pearson *et al.*, 1991). Although the release of another isocyanate from its parent GSH conjugate has been documented (Mutlib *et al.*, 1990), the high reactivity of methyl isocyanate has prevented its formal detection during metabolism of NMF. Nevertheless, detection of glutathione and cysteine conjugates of the involved isocyanate has been taken as empirical evidence of intermediate formation of these reactive compounds during metabolism. Free isocyanates preferentially react with cysteine (Brown *et al.*, 1987), but other nucleophilic centres may also be targeted, as demonstrated by carbamylation of the N-terminal valine in haemoglobin by MIC (Lee,

1976).

SMG and SMC are cytotoxic to isolated mouse hepatocytes and are potent inhibitors of TLX5 lymphoma cell growth (Han *et al.*, 1990). TLX5 cells, which are deficient in glutathione, are much more sensitive to SMG or SMC than to the parent formamide, N-methylformamide (Bill *et al.*, 1988). Excess glutathione (10 mM) significantly antagonized the deleterious effects of SMG and SMC on hepatocytes and lymphoma cells (Han *et al.*, 1990).

Evidence obtained with alkyl and benzyl isothiocyanates indicates that these compounds, which have been shown to release free isothiocyanates, damage cell membranes (Bruggeman *et al.*, 1988). It has also been shown that methyl ester derivatives of SMG and SMC do not demonstrate a marked difference in toxicity compared to SMG or SMC (Han *et al.*, 1990). Although glutathione and glutathione conjugates penetrate cell membranes to only a very limited extent, esters of glutathione are readily transported into cells (Meister, 1988). These facts, taken together with preliminary evidence that cellular depletion of GSH did not potentiate the toxicity of SMG in cell cultures (Han *et al.*, 1990) would suggest that SMG induces toxicity primarily by releasing methyl isocyanate at cell membranes.

1.5 Reproductive toxicology

In toxicology, chemicals and drugs are frequently classified according to which system they target, for example, the respiratory, immune, reproductive or CNS systems. Toxins may act on subcellular components, cells or organs. Commonly used indices of toxicity include gross pathology, histology, hormone and enzyme levels or activities, tissue growth, DNA damage and repair, pregnancy outcome and postnatal growth. It is

with such measures that the toxicity of various industrial chemicals has been documented.

A chemical could be toxic to the reproductive system by affecting any stage, from oogenesis and spermatogenesis to weaning of the F_1 generation and maturation of the offspring. The potential of a chemical to be a reproductive poison may be assessed in several ways: effects on gametes, sex hormone levels, fertilization and implantation efficiency, the oestrus cycle, foetal and postnatal growth and the foetal sex ratio. Both *in vivo* and *in vitro* tests are used to investigate mechanisms.

Lead, mercury, vinyl chloride, dimethylformamide, methyl butyl ketone, dibromochloropropane, carbon disulphide and various pesticides are some examples of human reproductive toxins to which people have been exposed, either in industry or via environmental contamination. The detrimental effects of these chemicals on human reproductive function are discussed in detail by Nisbet and Karch (1983).

1.5.1 Models for reproductive toxicity testing

In vivo administration of a chemical agent is the first step to document animal toxicity. This is the initial phase in reproductive studies as outlined by the Food and Drug Administration (FDA) of the United States and also adopted in Canada and Japan. Rodents are the most commonly used species. A very efficient gauge of female reproductive toxicity is the effect of a chemical on pregnancy development. The agent is administered to the female and the outcome of pregnancy followed. In this way one can observe the effects any chemical may have on the ability of the female to produce gametes, mate, conceive, maintain pregnancy, deliver viable young and nurture them until weaning.

For purposes of analysis, especially pharmacokinetic studies, it is usual to examine effects of chemicals on the pregnant female based on whether they target the mother, the placenta or the foetus (Miller, 1983). If severe enough, maternal toxicity will inevitably result in foetal toxicity due to the dependence of the foetus on maternal homeostasis for normal growth.

Foetal toxicology examines indirect or direct effects of chemicals on the development of the foetus. If the effects result in an abnormal phenotype, the chemical is classified as a teratogen. Direct foetal toxicity implies toxic action at the level of the embryo. The rodent embryo culture system developed by New (New, 1966; New *et al.*, 1976) has become the standard method of isolating the embryo from maternal influences to examine direct foetal toxicity. It is particularly useful because the stage of development in which mouse and rat embryos can be successfully cultured occurs during the period of organogenesis, when the embryo is most susceptible to teratogenic insult. Embryos can be cultured *in vitro* for about 48 hours and demonstrate comparable growth and development to embryos *in vivo*. Other methods have been developed to look at effects of chemicals on specific foetal organs, such as limb buds (Kochhar, 1973) or palatal shelves (Saxen, 1966). More recently, the trend to reduce whole animal use has resulted in cultures of cells derived from various foetal organs (Welsh, 1987).

Indirect foetal toxicity could be the result of chemical action toxic to the mother or to the placenta. Cultures of rodent (Williams *et al.*, 1975a, 1975b) and human (Miller *et al.*, 1985) placenta enable analysis of placental function isolated from both maternal and foetal influences. Combinations of these methods allow one to conclude whether a toxin acts solely on the foetus or mother or if adverse effects are the result of the toxin acting at several sites.

1.5.2 Role of the yolk sac placenta

The yolk sac is the placenta of rodents early in organogenesis. Its functions are gradually replaced by those of the developing chorioallantoic placenta during mid-gestation (Jollie, 1990). The role of the yolk sac in mediating embryonic nutrition is well established. Uptake is the rate-limiting step in the yolk sac nutritive pathway (Williams *et al.*, 1975a,b). Uptake occurs by fluid-phase pinocytosis, which is a constitutive activity, and by receptor-mediated endocytosis, which generally takes up large molecular weight substrates at many times the fluid-phase rate. Once taken up, proteins are degraded within yolk sac lysosomes to amino acids, then delivered to the embryo for subsequent protein synthesis. Lysosomal degradation of proteins by the yolk sac provides more than 99% of the amino acids used by the embryo for protein synthesis early in gestation (Freeman *et al.*, 1981; Beckman *et al.*, 1990).

Recognition of the yolk sac as the site of action of a teratogen can be dated to 1940, when Seegal and Loeb reported that injection of antiplacental serum into pregnant rats resulted in an increase in resorptions and degeneration of foetal organs (Seegal and Loeb, 1940). In the 1960's and early 1970's rat teratogenic antisera raised against placenta or kidney, which cross-reacts with the placenta, was shown to act at the level of the yolk sac (Brent, 1964; Slotnick and Brent, 1966; Brent *et al.*, 1971). Although all yolk sac antisera localize to the yolk sac, only those that inhibited pinocytosis were shown to be teratogenic (Lerman *et al.*, 1986). It has also been shown that inhibition of yolk sac lysosomal proteolysis, by direct inhibition of lysosomal enzymes, can also be teratogenic (Beck and Lowy, 1982; Freeman and Lloyd, 1983b; Daston *et al.*, 1991).

1.5.3 Examples of foetal toxins

The best known example of a direct-acting foetal toxin is probably the teratogen thalidomide (Schardein, 1976). Ingestion by the mother during days 21-36 of the first trimester resulted in phocomelia and amelia. Organic mercury is an example of an environmental contaminant that led to abnormal offspring in humans (Koos and Longo, 1976), probably via direct actions on the embryo *in utero*. Ethanol ingestion by the mother during pregnancy can result in development of the foetal alcohol syndrome (FAS) (see Clarren and Smith, 1978). Ethanol does affect placentation and maternal blood supply but it has also has been shown to directly affect foetal brain development, delaying synaptogenesis and reducing plasticity (Volk, 1984; Hoff, 1988).

An example of an indirect foetal toxin is salicylate. Salicylate is excreted as a sulphate conjugate and the reduction in sulphate levels after maternal ingestion of salicylate leads to depleted foetal levels of sulphate and so reduced synthesis of glycosaminoglycans in the foetus, resulting in teratogenesis (Knight *et al.*, 1978). Cadmium is an example of an industrial agent causing foetal toxicity by actions at the level of the yolk sac at concentrations which are not toxic to either the mother or the foetus (Levin and Miller, 1980).

CHAPTER TWO
MATERIALS AND METHODS

2.1 Chemicals

The following agents were purchased: monomethylamine hydrochloride, dimethylamine hydrochloride, trimethylamine hydrochloride, trimethylamine-N-oxide, L-cysteine, D-cysteine, N-acetylcysteine, glutathione, yeast RNA (Type XI), calf thymus DNA (Type I), bovine serum albumin (Fraction V), aminooxyacetic acid, leupeptin, orcinol, diphenylamine, acivicin, (-)-2-oxo-4-thiazolidine carboxylic acid (Sigma Chemical Co., St. Louis, MO); vitamin A palmitate, ascorbic acid, zinc acetate (BDH Chemicals, St. Laurent, Quebec); Hank's balanced salt solution, Bigger's medium, Tyrode's saline, penicillin-streptomycin, Medium 199 (Gibco BRL, Burlington, Ontario); IGF-II (Smithkline Beecham, King of Prussia, PA); insulin (Eli Lilly Co., Indianapolis, IN); methyl isocyanate (Aldrich Chemical Co., Milwaukee, WI);^{3} H-Thymidine (53 Ci/mmol), ^{3} H-uridine (37 Ci/mmol), ^{3} H-leucine (64 Ci/mmol), L-^{3}H-leucine (131 mCi/mmol) and ^{125}I-bovine serum albumin (1 mCi/mg) (ICN Biomedicals, St. Laurent, Quebec), ^{14}C-sucrose (632 mCi/mmol)(DuPont, Mississauga, Ontario) and all other chemicals of high purity grade (Fisher, Montreal, Quebec). S-(N-methylcarbamoyl)glutathione was a gift from Dr Thomas Baillie, University of Washington, Seattle, WA.

2.2 Animals

Swiss CD-1 mice (20-25 g) and Sprague-Dawley male rats (175-200 g) were purchased from Charles River Canada, St. Constant, Quebec. Animals were housed in the Animal Resources Centre of McGill University. Animals were kept on a 12 hour light 12 hour dark

cycle (lights on at 07.00), in temperature (22-25°C) and humidity (50-70%) controlled facilities, in Plexiglass cages lined with heat-treated woodchip bedding (Northeastern Products, Warrensburg, New York). Animals had free access to mouse or rat chow pellets (Purina, St. Louis, MO.) and water. The protocols and use of animals were approved by the McGill University Animal Care Committee.

For mating, one male mouse was placed in a cage with 4 females for two hours between 07.00-09.00 hours; the presence of vaginal plugs indicated day 0 of gestation.

2.3 Labelling of serum proteins

Male rats were injected via the tail vein with ^{3}H-leucine (100 μCi/100 g body weight). Two hours later, rats were anaesthetized with ether and 10-15 ml blood collected by cardiac punctures. The serum was immediately separated, pooled, and placed into dialysis tubing (Spectrapor, 12-14,000 MW cutoff; Spectrum, Houston, TX). The serum was dialysed at 4°C for 48 hours in 2.5 litres of Hank's balanced salt solution which was changed at 24 hours; the dialysate medium was counted periodically and there was a minimal increase in radioactivity between 24-48 hours. Serum in the dialysis bag was heat inactivated, filter sterilized (Acrodisc, Gelman, Ann Arbor, MI) and stored at -20°C until use. The serum contained 1,500,000 dpm/ml and > 99% of the radioactivity was precipitable by TCA. For embryo culture, the media consisted of 75% ^{3}H-leucine-labelled serum: 25% unlabelled serum.

2.4 Polyacrylamide-gel electrophoresis of serum

Samples of ^{3}H-leucine-labelled serum were separated on a 10% polyacrylamide gel, using a BIO-Rad Mini Gel System (Bio-Rad, Richmond, CA). The gel was scanned for

density using an LKB Ultrascan and lanes were cut into sections, solubilized and radioactivity counted. The band corresponding to albumin contained 70 ± 1% of total protein and 44 ± 1% of total radioactivity (mean ± SEM of 3 separate lanes).

2.5 *IN VIVO* TOXICITY

2.5.1 Exposure to methyl isocyanate (MIC)

MIC was administered by whole-body exposure as previously described (Ferguson *et al.*, 1986; Varma *et al.*, 1987). The exposure chamber was a glass 37 litre tank (approximate dimensions 50 x 30 x 25 cm) with a removable 12 mm thick Plexiglas lid. The bottom of the tank was lined with a removable stainless steel grid so that urine and faecal matter dropped under the grid. The lid had three holes (15 mm diameter) centrally located in a line at the centre. The free end of a 75 cm long polyethylene tube (PE 190) connected to the bottle containing the methyl isocyanate (MIC) was anchored into one side hole in the lid such that the open end of the tube hung approximately 8 cm below the lid.

The exposure chamber was exhausted by a vacuum pump at a minimum flow rate of 30 litres per minute, monitored by a Lurex flowmeter. The centre hole in the tank lid was sealed with a Teflon-coated rubber septum and was used to draw samples for the assay of MIC. To deliver MIC into the chamber, dry air was passed into a 240 ml bottle containing 2-3 ml liquid MIC via an 18 gauge 50 mm steel hypodermic needle inserted through the Teflon-coated septum of the bottle cap. MIC vapour escaped through another hypodermic needle with the sharp end projecting out of the bottle cap; to this second hypodermic needle a polyethylene tube was connected that carried MIC vapour into the exposure tank. The exhausted MIC from the exposure tank was passed through a charcoal filter and then into a flask containing 5 M sodium hydroxide before being discharged into the fume hood

exhaust.

Prior to the start of the exposure, the concentration of MIC in the chamber was adjusted either by changing the air inflow rate, which diluted the MIC concentration, or by changing the MIC inflow. After the MIC concentration had stabilized to the desired level, the MIC delivery tube was temporarily taken out of the exposure chamber, the animals placed inside, and the tube replaced. MIC concentration in the chamber was assayed at 10 to 15 minute intervals and did not alter by more than 1 ppm during the entire course of the experiment. When set to deliver 9 ppm and 20 ppm, the concentrations were 9 ± 0.3 and 20 ± 0.8 ppm, respectively.

Two approaches were adopted in an attempt to determine if maternal and foetal toxicities of MIC could be dissociated. In the first approach, mice were exposed on day 8 of gestation to 20 ppm MIC for 2 hours. The exposure was done once, to partly simulate the situation in Bhopal when MIC was released. This gestational age was selected because it corresponds to the time period most suitable for *in vitro* embryo culture (New, 1966). Immediately after the end of exposure, mice were killed by cervical dislocation, the abdomen opened and the uterine horns transferred to sterile 0.9% saline. Embryo culture was then carried out as detailed below.

In the second approach, embryos from untreated day 8 pregnant mice were removed and placed into culture bottles as described below. MIC vapour was then injected into the dead space of the sealed culture bottles so as to produce concentrations ranging from 714 to 2144 ppm inside the bottle. The culture then proceeded as for controls and embryonic development was recorded at 42 hours, according to Brown and Fabro (1981).

2.5.2 MIC Assay

MIC was assayed according to the procedure developed by Ferguson *et al.* (1986). A Hewlett Packard gas chromatograph (model 5890A) was used, fitted with an OV-210 packed column and a nitrogen-phosphorus detector, connected to a Hewlett Packard integrator (3380A). The concentration of MIC in the exposure chamber was compared to that of the standard on the basis of peak area. The injection temperature was 200°C, the oven temperature 60°C and the detector temperature 250°C. Helium was used as the carrier gas, at a flow rate of 20 ml/minute (inlet pressure 25 psi), hydrogen at 4 ml/minute and dry air at 100 ml/minute. The retention time of MIC under these conditions did not vary by more than 0.01 minute during the exposure and was 0.76-0.81 minutes on different days.

2.5.3 Administration of methyl isocyanate metabolites

For an acute exposure to monomethylamine, dimethylamine, dimethylurea and trimethylamine, these compounds were dissolved in 0.9% saline and injected intraperitoneally on day 8 of gestation. SMG was dissolved in double distilled water and injected in a volume of 0.1 ml at doses of 100 mg/kg and 500 mg/kg. Initial experiments indicated that SMG at 100 mg/kg had no effect on pregnancy outcome. Therefore, prior depletion of GSH was attempted by buthionine sulfoxime (BSO) treatment 90 minutes before SMG. The control group of mice received BSO (900 mg/kg) alone; the treated groups received both SMG and BSO.

For chronic exposure to methylamines, the total daily dose of TMA was injected intraperitoneally. Initially, the dose was given only once, between 07.00 hours and 08.00 hours. However, at 5 mmol/kg TMA, significant mortality occurred, so in later studies TMA was administered in two equally divided portions, once between 07.00 - 08.00 hours and

then between 16.00-17.00 hours from day 1 to 17 of gestation or from day 6 to day 15. Controls were injected with 0.1 ml saline, the volume used to inject TMA.

2.5.4 Assessment of pregnancy outcome

Dams were killed by cervical dislocation on day 18 of gestation, the foetuses delivered by cesarean section and various parameters measured. In the absence of obvious signs of implantation, uteri were stained with 10% ammonium sulphide to identify implantation sites (Abel and Tan, 1987). Pup weights for each female were calculated by dividing the sum of the body weights of all live pups in a litter by the number of live pups. Mean pup weights for each treatment group was based on the sum of mean pup weight for each female divided by the number of females in the group. Litter size denoted only live foetuses. Foetuses were killed by excess ether then randomly placed either in Bouin's solution for visceral examination by the freehand razor sectioning technique (Wilson, 1965) or in 95% ethanol, followed by clearing in potassium hydroxide for skeletal examination by the alizarin red S staining technique (Dawson, 1926).

2.5.4.1 Adverse reproductive index

Existing quantitative assessment of reproductive toxicity is based on comparing each variable in the treated group to the same variable in controls but does not cumulate all expressions of toxicity into one unit for comparison with a similar unit derived from control animals. We used a system termed adverse reproductive index (ARI), in addition to standard methods, to assess overall reproductive performance based on maternal body weight and mortality as well as foetal and placental weights, resorptions and foetal deaths. Each variable was allotted an arbitrary number as follows: maternal death, 10; decrease in maternal weight

gain relative to controls, 2.5 for 1 SD (standard deviation), 5 for 2 SD, 10 for 3 SD; implants lost, 0.5 for each implant lost or 5 for 100% loss (based on observed normal litter size of 10); dead foetuses, 0.5 for each dead foetus and 5 for all foetuses dead; decrease in mean foetal body weight of the litter relative to controls, 2.5 for 1 SD, 5 for 2 SD, 7.5 for 3 SD; decrease in placental weight relative to controls, 0.5 for 1 SD, 1 for 2 SD, 2 for 3 SD. The scores for each animal were added to calculate mean scores for the group of control mice and for mice treated with different doses of methylamines. Although an estimation of ARI does not indicate the mechanism or site of toxicity and assumes maternal death as a far greater expression of toxicity than any other variable, it may be a reasonable expression of overall reproductive toxicity of a chemical.

2.5.4.2 Postnatal growth

Litters were randomly culled to 8 pups 24 hours after birth. Body weights were recorded at birth, at 24 hours after birth and weekly thereafter for up to 14 weeks. Pups were weaned at three weeks and housed separately according to their sex. At 3 and 8 weeks after birth, a small number of animals from each litter were decapitated. Various organs were removed, weighed and then stored at -80°C for measurements at a later date of protein and DNA as described below. The trunk blood was collected from only pups in the 7.5 mmol/kg/day litters. Sera from all male mice caged together (maximum 4) were pooled to yield one sample and testosterone was measured using a radioimmunoassay kit (Immunocorp, Montreal, Canada).

2.6 *IN VITRO* TOXICITY

2.6.1 Embryo culture

On the morning of day 8 of gestation, mice were killed by cervical dislocation and embryos harvested for culture according to the method of New (1966). Uteri were removed from the dam, rinsed in sterile 0.9% saline, then placed in Tyrode's saline, where decidua were removed from the uteri. Embryos with decidua were transferred to sterile Hanks balanced salt solution (pH 7.4). After removing decidua and Reichert's membrane, embryos were placed at 37°C in heat (56°C for 30 min)-inactivated rat serum diluted with Tyrode's saline in a ratio of 9:1. After all embryos from all the animals on a given day were removed (usually 2 hours), they were placed in prewarmed 60 ml culture roller bottles filled with 1.6 ml of a mixture of serum-Tyrode's solution for each embryo; culture media contained 50 U penicillin and 50 µg streptomycin per ml. Usually 4-5 embryos were placed in one bottle. The media was gassed with a mixture of 5% O_2, 5% CO_2 and 90% nitrogen for 2 minutes; bottles were capped and placed on their side on a rotor (60 rpm) maintained at 37°C. The following morning and evening, embryos were gassed again with a mixture of 5% CO_2 and 95% air.

Embryos were scored according to Brown and Fabro (1981) at the end of each labelling period or at 42 hours of culture, using a binocular microscope. The presence of a yolk sac circulation and vigorous heart beat were used as criteria for further processing, which was done as follows. Embryos were separated from yolk sacs, washed three times in ice-cold saline and biochemical analysis was either performed on the same day or embryos were stored at -80° C for analysis at a later time (no longer than 3 weeks).

2.6.2 Exposure of embryos to methylamines and SMG

Embryos were allowed to equilibrate for one hour in the culture bottles before any chemical was added; the end of this period was considered time zero. Methylamines and SMG was dissolved in autoclaved double distilled water immediately prior to addition to the culture bottles. Initially, the effects of increasing concentrations (0.1 to 2 mM) on embryonic development was determined. Following these initial studies, concentrations of 0.75 mM TMA and 0.25 mM SMG were chosen for the remainder of the experiments, unless otherwise indicated. These concentrations reduced growth of treated embryos to approximately 75% of untreated embryonic growth levels at 42 hours. No significant mortality was associated with SMG at 0.25 mM; TMA at 0.75 mM led to 30% mortality.

2.6.3 Treatment with antioxidants and thiol compounds

To determine the effects of antioxidants, 100 µM L-cysteine, D-cysteine, glutathione and L-ascorbic acid were added to the media just prior to addition of TMA and embryos scored at 42 hours. Controls for these experiments were treated with each of the antioxidants without TMA. Effects of several concentrations of L-cysteine on TMA toxicity were also determined. In order to determine if SMG toxicity can be modified by thiol donors, 1-3 mM glutathione (GSH) and 1-6 mM of the cysteine prodrug (-)-2-oxo-4-thiazolidine-carboxylic acid (OTC) were added simultaneously with SMG to cultures and effects of these treatments on embryo development determined. L-Cysteine was toxic to embryos at 1 mM concentration and therefore was not used.

2.6.4 Treatment with growth factors

Insulin and growth factors stimulate embryonic growth (King *et al.*, 1981) and deficiencies of vitamin A and zinc are associated with growth deficiency (see Wasserman and Corradino, 1971; Favier, 1992). We therefore determined if TMA toxicity could be modified by growth factors such as insulin and IGF-II and by vitamin A and zinc. Insulin, IGF-II, vitamin A and zinc were added to embryos in culture, 30 minutes before the addition of TMA. At the end of culture embryos were scored for growth parameters and protein content.

2.6.5 Osmolarity and pH measurements

In order to find out if the toxic effects of TMA on embryos were due to changes in osmolarity, sucrose and glycerol were added to the culture media at the beginning to achieve a final concentration of 1 and 2 mM. Because TMA is a weak base, the pH of the culture medium in the absence and the presence of 0.75 mM TMA was measured at the beginning and end of the culture period.

2.6.6 Uptake of labelled thymidine, uridine and leucine

Embryos were allowed to equilibrate for 1 hour after starting the culture; the end of this hour was designated time zero. At 0, 2, 6, 14, 22 and 40 hours, ^{3}H-thymidine, ^{3}H-uridine or ^{3}H-leucine was added to the culture medium at concentrations of 2 μCi/ml; the labelling period was 2 hours. ^{3}H-Thymidine and ^{3}H-uridine were added directly to the normal culture medium (rat serum: Tyrode's saline 9:1). However, for the determination of leucine incorporation into proteins, embryos were transferred at the above listed times from the normal culture media to a protein labelling media, the composition of which was as follows: To 90 ml of physiological salt solution was added 10 ml of heat-treated rat serum, 10

µmoles glucose and 2 µmoles of each of the following amino acids: alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glycine, glutamine, histidine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine. This media is a modification of that used by Alter *et al.* (1976) in so far as unlabelled leucine was not added but 10% rat serum was needed for embryonic growth. Following the 2-hour pulse labelling, macromolecules were isolated as described below.

2.6.7 Quantification of macromolecules

2.6.7.1 Labelled macromolecules

For extraction of the RNA fraction embryos (2-4 pooled if < 24 hours old, singly if > 24 hours) were sonicated on ice for 90 seconds in polyethylene tubes containing 0.5 ml phosphate buffered saline (PBS). One ml of 0.3 N perchloric acid (PCA) was added to each tube, followed by vortexing then centrifugation at 15,000 g for 5 minutes. The supernatant was discarded, 0.5 ml of 0.3 N KOH was added and the tubes heated to 37°C for one hour. Tubes were cooled on ice, 1 ml of 0.3 N PCA was added followed by centrifugation at 25,000 g for 5 minutes. From the supernatant 0.5 ml in duplicate was removed for RNA determination by the orcinol reaction (Schneider, 1957), using yeast RNA, type XI as standard. From the same supernatant 0.2 ml, in duplicate was removed and placed in 20 ml plastic scintillation vials to which was subsequently added 6 ml of Formula 963 scintillation fluid (NEN Research Products, Boston, Massachusetts). Thoroughly vortexed vials were counted in a Wallac 1410 liquid scintillation counter (Pharmacia, Turku, Finland).

Embryonic DNA fractions were extracted as follows. Initial treatment of embryos was exactly the same as for embryos from which RNA was extracted, up until the removal of the supernatant following the first centrifugation. At this point 0.6 ml of 10% trichloroacetic acid

(TCA) was added to each tube, followed by heating to 80°C for 30 minutes. The tubes were cooled on ice then centrifuged at 3000 g for 5 minutes. Aliquots (0.05 ml), in duplicate, were removed for quantification of DNA by the diphenylamine reaction (Burton, 1956), using calf thymus DNA as standard. Aliquots (0.2 ml), in duplicate, were taken from the supernatant and added to 20 ml plastic scintillation vials, to which was added 6 ml of scintillation fluid. The vials were placed in the scintillation counter and counted as for the RNA fractions.

The extraction of embryonic protein fraction was based on that of Beckman *et al.*, (1990). Embryos (2-4 pooled when < 24 hours old) were homogenized in 1 ml of PBS using a Polytron (Brinkman Instruments, Rexdale, Ontario, Canada) for 30 seconds at level 6. Of the homogenate, 0.2 ml was removed and added to 1.8 ml of 0.5 N NaOH, placed in a shaking water bath at 30°C overnight, then assayed for protein content the following day by using duplicate 0.2 ml portions of this homogenate/NaOH mixture in the protein dye binding assay of Bradford (1976). Bovine serum albumin was used as standard. The remaining 0.8 ml of homogenate was incubated with 1.2 ml of TCA (final concentration 7.5%) for 1 hour at 4°C. The tubes were then centrifuged at 3,000 g for 10 minutes, the supernatant discarded, the pellet washed three times with diethyl ether, air dried and dissolved in 0.5 ml of 0.5 N NaOH. After 30 minutes at room temperature 0.2 ml, in duplicate, was removed and placed into 20 ml plastic scintillation vials. To these were added 0.6 ml of glacial acetic acid and 8 ml of scintillation fluid. Counting of radioactivity then proceeded as for the DNA and RNA fractions.

2.6.7.2 Unlabelled macromolecules

Following morphologic scoring, embryos and yolk sacs were separated and processed in an identical manner. Usually, 2-4 embryos cultured for less than 24 hours were pooled

for analysis. Embryos were rinsed 3 times in ice-cold saline and then homogenized in 1 ml of phosphate buffered saline (pH 7.4). From this homogenate, 200 µl were removed for protein determination after 5-fold dilution and overnight incubation at 30°C in 0.5 N sodium hydroxide; bovine serum albumin was used as standard. To the remaining 800 µl of the homogenate was added 1 ml of 0.3N perchloric acid. Tubes were centrifuged for 5 minutes at 25,000 x g, the supernatant was discarded and 1 ml of 10% trichloroacetic acid (TCA) was added for the extraction and assay of DNA. The TCA homogenate was incubated at 80°C for 30 minutes, then cooled on ice and the tubes were centrifuged at 3,000 x g for 5 minutes. From the supernatant, two 100 µl aliquots were removed and radioactivity was counted on a liquid scintillation counter (LKB 1219) to determine ^{3}H-thymidine uptake. From the remaining supernatant two 300 µl aliquots were removed for determination of DNA by the diphenylamine method (Burton, 1956) using calf thymus DNA as standard.

2.6.8 Yolk sac culture

Pregnant mice were killed on day 15 of gestation. Uteri were removed and transferred to cold Tyrode's saline and yolk sacs were separated from embryos. Individual yolk sacs were placed in the culture media at 37°C for 30 minutes preincubation followed by 30 minutes of incubation in the presence of TMA or SMG; the end of this time point was considered time zero. Radiolabels were then added. For measurement of fluid phase pinocytosis, yolk sacs were cultured in 5 ml of culture media (90% Medium 199: 10% rat serum). For measurement of receptor-mediated uptake, yolk sacs were cultured in 10 ml of Medium 199.

2.6.8.1 Fluid-phase pinocytosis

^{14}C-Sucrose was added to embryo cultures 18 hours after the addition of TMA or at time zero in the case of isolated yolk sacs. At hourly intervals for 5 hours, embryos or yolk sacs were removed from the culture bottles; day 9 yolk sacs were dissected free of embryonic tissue. Yolk sacs were washed three times with saline and then digested overnight in 2 ml of 0.25 N NaOH at 30°C. The following morning, the digestants were diluted with an equal volume of 0.25 N HNO_3. Duplicate 50 µl aliquots were removed for protein determination. Duplicate 0.45 ml portions were removed, placed in 6 ml scintillation vials with 4.5 ml of scintillation fluid and the radioactivity counted on a beta-counter (LKB 1219 Rackbeta).

2.6.8.2 Receptor-mediated pinocytosis

At time zero, 10 µl of ^{125}I-BSA solution (1 µCi) was added to the yolk sac cultures. At hourly intervals for 5 hours, duplicate 0.5 ml samples of media were removed and added to 0.5 ml volumes of 80% medium 199:20% serum prior to the first count. Yolk sacs were removed at 5 hours, rinsed 3 times with saline and then homogenized by Polytron in 5 ml of sterile water. One ml portions were removed for protein assay. Duplicate 1 ml volumes of the homogenates were counted with the initial media samples on a gamma counter (LKB CliniGamma 1272). After the initial count, 1 ml of 20% trichloroacetic acid was added to all tubes, followed by centrifugation (3000 g, 10 minutes.). One ml aliquots of supernatant were removed from all tubes and recounted as a measure of TCA-soluble radioactivity.

2.6.8.3 Quantification of uptake and lysosomal degradation

Uptake of ^{14}C-sucrose and ^{125}I-BSA was calculated according to the method of Livesay and Williams (1979). Briefly, uptake of ^{14}C-sucrose was calculated by dividing the value for

total radioactivity per yolk sac by the specific activity of ^{14}C-sucrose and converting this to ng. Data is expressed as ng ^{14}C-sucrose/mg yolk sac protein.

Uptake of ^{125}I-BSA, Q (ng ^{125}I-BSA/mg yolk sac protein), was calculated from the following formula (Livesay and Williams, 1979):

$$Q = \frac{S}{P} \times \frac{(T+Y)}{M}$$

where S is the initial concentration (ng/µl) of ^{125}I-BSA in the culture medium, P is the protein content of the yolk sac (mg), T is the total amount of TCA-soluble radioactivity (cpm) appearing in the culture medium during the incubation period, Y is the radioactivity (cpm) associated with the yolk sac at the end of the incubation period and M is the mean quantity of TCA-insoluble radioactivity (cpm/µl) in the medium over the incubation period.

Lysosomal degradation was determined by measuring TCA-soluble radioactivity, which appeared in the culture medium after addition of ^{125}I-BSA to the cultures. Leupeptin has been shown to inhibit lysosomal degradation (Knowles *et al.*, 1981) and was used as a positive control.

2.6.9 Limb bud cultures

The culture method was based on that of Kochhar (1973) as modified by Hales and Jain (1986). Dams were killed on day 12 of gestation. Uteri were removed and transferred to Tyrode's saline and embryos dissected free of maternal tissues. Embryos were transferred to Bigger's medium and fore as well as hind limbs were removed and pooled. Limb pairs (one fore and one hind limb) were placed in 60 ml culture bottles containing 3 ml of Bigger's medium and 1 ml of rat serum. The culture medium was gassed with 20% O_2:5% CO_2:75% N_2 and incubated at 37°C in a rotator at 60 rpm. At 17.00 hours the following day

(approximately 30 hours after the start of culture) 1 μCi/ml ^{3}H-leucine was added for a 16 hour labelling period. At the end of this time, pairs of limbs were rinsed three times in saline then homogenized in 600 μl of distilled water. One 100 μl aliquot was removed for the measurement of total radioactivity; to the remainder was added 300 μl of 20% TCA and the tubes kept at 4°C for 1 hour followed by centrifugation at 3,000 g for 10 minutes. The supernatants were discarded, the pellet dried under air for 1 hour and then dissolved in 500 μl of 1 N NaOH at 30° for 3 hours. From these tubes, 400 μl samples were removed and counted for radioactivity after neutralization with 500 μl of 1 N HNO_3. Duplicate 25 μl aliquots of the remaining 100 μl were utilized for protein assay.

2.7 Protein assay

Protein was determined by the dye-binding method (Bradford, 1976) with bovine serum albumin as the standard. Unless otherwise noted, tissue homogenates for this purpose were digested in sodium hydroxide solution overnight at 30°C.

2.8 Statistics

Data were analyzed by Student's *t*-test or the Chi-square test. Multiple comparisons were made by one-way analysis of variance, followed by the Bonferroni test for significance. A probability of less than 0.05 was assumed to denote significant difference. Where applicable, data are presented as mean ± standard error of the mean.

CHAPTER THREE
RESULTS

3.1 *IN VIVO*

3.1.1 Behaviour of mice exposed to methyl isocyanate (MIC)

Mice exposed to MIC vapour in the glass exposure chamber usually ceased their exploratory behaviour after several minutes, rubbed their noses and adopted a crouching posture with noses held close to the floor. They remained immobile with closed eyes unless disturbed.

3.1.2 General toxicity of methylamines

At 0.25, 1 and 2.5 mmol/kg dose levels, monomethylamine (MMA) and dimethylamine (DMA) did not produce any apparent behavioral changes or gross toxic effects. Trimethylamine (TMA) at a dose level of 2.5, 5 and 7.5 mmol/kg and MMA and DMA at 5 mmol/kg caused contractions of the abdominal muscles in the proximity of the injection site. These contractions lasted 2-3 minutes after MMA and DMA and 10 minutes after TMA. Within 3 minutes after injection of TMA, mice became ataxic, the breathing became shallow and rapid (not quantified), there was nasal discharge and hypersalivation and tremors developed. Animals responded to touch and the righting reflex was not lost. Death was preceded by convulsions. These effects of DMA and TMA were neither sex nor pregnancy dependent. The symptoms lasted for approximately 10-30 minutes and if the animal did not die during this period, complete recovery occurred within 1 hour.

A dose of 5 mmol/kg/day TMA caused the death of 5 of 11 pregnant and 6 out of 6 virgin mice. The death of pregnant mice (pregnancy ascertained at necropsy) was not

preceded by an effect on body weight. However, virgin mice steadily lost weight; 4 animals died on day 6 of treatment and all had died by day 9.

3.1.3 Effects of methylamines on pregnancy outcome

3.1.3.1 Single dose

Intraperitoneal injections of 0.3, 1 and 3 mmol/kg MMA, DMA, TMA or dimethylurea (DMU) did not produce any observable toxicity in either the dams or the foetuses (Table 1). There was no difference in body weight gain of dams in the treatment groups compared to controls nor in all recorded parameters of pregnancy outcome.

3.1.3.2 Multiple doses

The effects of MMA, DMA and TMA administered from day 1 to day 17 of gestation on pregnancy outcome are presented in Tables 2, 3 and 4, respectively. At all dose levels studied, MMA and DMA did not exert any significant effect on pregnancy outcome. TMA at 2.5 and 5 mmol/kg caused a significant decrease in foetal weight without affecting maternal body weight gain. Because of a relatively greater decrease in foetal than placental weights, ratios of placental to foetal weights increased in mice treated with 2.5 and 5 mmol/kg TMA. Numbers of resorbed and dead foetuses were equally distributed across all doses of MMA and all but the highest dose of DMA and TMA. The number of resorptions but not the number of dead foetuses was significantly greater in mice treated with 5 mmol/kg DMA than in control animals. On the other hand, at 5 mmol/kg TMA, the number of dead foetuses but not the number of resorptions was significantly greater than in controls. None of the amines caused a significant increase in external, internal organ or skeletal abnormalities.

Table 1. Effect of a single injection of methylamines and methylurea on pregnancy development in mice

Variables	Saline	MMA	DMA	TMA	DMU
Dams (n)	14	11	12	11	6
Day 1 BW (g)	24±1	25±1	25±1	24±1	25±1
Day 18 BW (g)	51±2	48±3	51±1	46±2	48±2
Implants lost (%)	8±3	12±9	20±3	12±9	9±7
Foetal BW (g)	1.3±0.04	1.3±0.03	1.4±0.03	1.3±0.02	1.3±0.03
PW (mg)	110±3	110±30	110±40	120±7	110±10
Litter size (n)	10.7±0.6	11.2±0.6	9.8±0.5	10.7±0.5	9.5±1.0

MMA (Monomethylamine), DMA (dimethylamine), TMA (trimethylamine) and DMU (dimethylurea) were injected (ip, 3 mmol/kg) on day 8 of gestation; mice were killed by cervical dislocation on day 18 of gestation. BW, body weight; PW, placental weight. Foetal and placental weights are mean weights per litter.

Table 2. Effect of monomethylamine on pregnancy development in mice

	Dose (mmol/kg/day)				
Variables	0	0.25	1.0	2.5	5.0
Dams (n)	29	7	8	6	8
Day 1 BW (g)	24.1±0.4	22.9±0.4	24.5±1.1	22.5±0.4	23.5±0.5
Day 18 BW (g)	48.4±1.1	46.3±1.7	48.5±3.6	44.9±1.4	46.8±1.4
Dams dead	0	0	0	0	0
Dead foetuses/litter	0.83±0.14	0.71±0.18	0.5±0.5	0.8±0.37	1.0±0.41
Resorptions/litter	0.66±0.13	0.29±0.18	0.5±0.22	1.4±0.4	0.71±0.29
Litter size (n)	9±1	8±1	11±1	9±1	10±1
Foetal BW (g)	1.4±0.03	1.3±0.04	1.3±0.05	1.4±0.05	1.3±0.05
PW (mg)	109±2	116±7	115±8	112±6	116±8
ARI	1.6±0.4	0.9±0.3	2.0±0.7	1.1±0.3	2.3±0.6

Mice were injected intraperitoneally, between 0700 and 0800 hours, from day 1 to day 17 of gestation. Mice were killed by cervical dislocation on day 18 of gestation. Foetal and placental weights represent mean weights per litter. BW, body weight; PW, placental weight. ARI (adverse reproductive index) represents cumulative maternal and foetal toxicity in arbitrary units.

Table 3. Effect of dimethylamine on pregnancy development in mice.

	Dose (mmol/kg/day)				
Variables	0	0.25	1.0	2.5	5.0
Dams (n)	29	9	13	11	11
Day 1 BW (g)	24.1±0.1	22.8±0.4	24.2±0.8	24.7±0.5	25.0±0.5
Day 18 BW (g)	48.4±1.1	48.9±1.0	50.1±2.2	50.9±1.5	45.9±2.0
Dams dead (n)	0	0	2	0	1
Dead foetuses/litter	0.83±0.14	0.78±0.32	0.63±0.26	0.86±0.4	1.29±0.36
Resorptions/litter	0.66±0.13	0.44±0.34	0.75±0.62	0.57±0.2	1.71±0.68*
Litter size (n)	9±1	9±1	10±1	10±1	8±1
Foetal BW (g)	1.4±0.03	1.5±0.05	1.4±0.03	1.4±0.04	1.4±0.03
PW (mg)	109±2	114±4	114±6	116±5	109±5
ARI	1.63±0.4	0.9±0.3	3.1±1.1	0.7±0.2	2.6±1.1

Mice were injected intraperitoneally, between 0700 and 0800 hours, from day 1 to day 17 of gestation. Mice were killed by cervical dislocation on day 18 of gestation. Foetal and placental weights represent mean weights per litter. BW, body weight; PW, placental weight. ARI (adverse reproductive index) represents cumulative maternal and foetal toxicity in arbitrary units. * Different ($p < 0.05$) from the corresponding control.

Table 4. Effect of trimethylamine on pregnancy development in mice.

	Dose (mmol/kg/day)				
Variables	0	0.25	1.0	2.5	5.0
Dams (n)	29	9	4	11	11
Day 1 BW (g)	24.1±0.4	23.4±0.5	23.1±0.4	23.4±0.5	28.2±0.9
Day 18 BW (g)	48.4±1.1	50.0±1.5	46.4±3.0	45.7±0.9	48.6±2.8
Dams dead	0	0	0	0	5*
Dead foetuses/litter	0.83±0.14	1.11±0.35	1.25±0.75	0.56±0.24	1.63±0.32*
Resorptions/litter	0.66±0.13	0.33±0.17	0.25±0.25	0.44±0.18	1.38±0.56
Litter size (n)	10±1	10±0	8±2	11±1	11±1
Foetal BW (g)	1.4±0.03	1.4±0.1	1.4±0.04	1.1±0.08*	1.0±0.06*
PW (mg)	109±2	118±5	116±9	103±4	98±5
ARI	1.6±0.4	1.0±0.3	1.5±0.4	4.3±1.1*	8.0±0.8*

Mice were injected intraperitoneally, between 0700 and 0800 hours, from day 1 to day 17 of gestation. Mice were killed by cervical dislocation on day 18 of gestation. Foetal and placental weights represent mean weights per litter. BW, body weight; PW, placental weight. ARI (adverse reproductive index) represents cumulative maternal and foetal toxicity in arbitrary units. * Different ($p < 0.05$) from the corresponding control.

3.1.4 Effects of TMA on the F1 generation

3.1.4.1 Growth

Injections of 1, 2.5, 5 and 7.5 mmol/kg TMA in two divided doses from day 6 to day 15 of gestation did not decrease maternal body weights. Foetal body weights were reduced at the 5 mmol/kg dose level and foetal body weights and litter size were reduced at the 7.5 mmol/kg dose level (Table 5). There was no maternal mortality in any treatment group. All animals delivered before 09.00 hours on day 19 of gestation. There was no apparent difference in the manner in which treated or control dams reared their young. The sex ratio at birth was not recorded; however, after random culling at 24 hours, there was no difference in male:female ratios of pups in any of the dosage groups.

Offspring of mice treated with 1 and 2.5 mmol/kg/day TMA from day 6 to 15 grew at the same rate as controls. At 5 and 7.5 mmol/kg TMA caused a greater inhibition in the body weight gain by male than by female offspring up to 8 weeks of age (Figure 2). At the highest dose, growth of female progeny was also reduced although this was significantly less than the effect on the male offspring (Figure 2).

Treatment of dams with TMA at a dose of 2.5 mmol/kg/day throughout gestation (day 1 to 17) resulted in a decrease in body weights of male but not of female progeny for up to 14 weeks, the longest duration of observation.

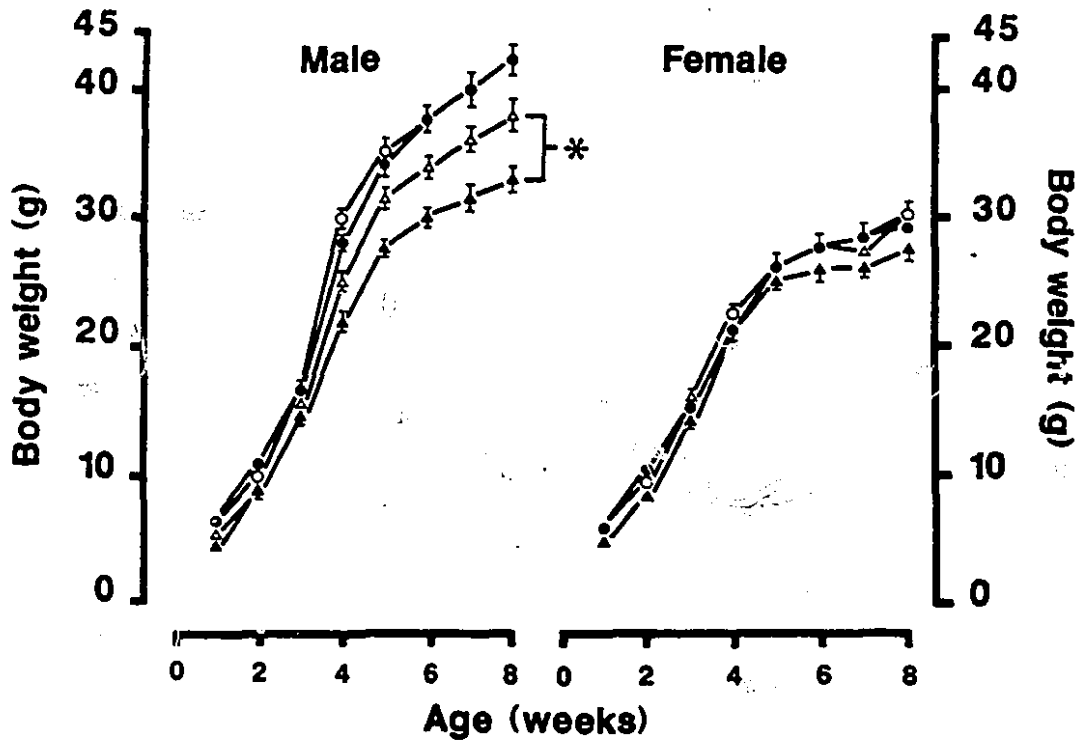

Figure 2. Body weights of offspring of mice treated with trimethylamine (TMA). Mice were injected intraperitoneally from day 6 to day 15 of gestation with saline (o) or with 2.5 (•), 5 (▵) or 7.5 (▲) mmol/kg/day TMA. Each data point represents the mean of at least 8 animals. Vertical lines represent ± SEM. Body weights of male progeny of mice treated with 5- and 7.5 mmol/kg/day TMA and of female progeny of animals treated with 7.5 mmol/kg/day trimethylamine are different ($p < 0.05$) from corresponding body weights of controls at all ages.

Table 5. Effects of trimethylamine (TMA) administered during organogenesis on pregnancy outcome in mice

	Dose of TMA (mmol/kg/day, day 6-15)				
Variables	0	1	2.5	5	7.5
Dams (n)	14	4	4	5	5
Day 0 BW (g)	23.4±0.6	22.7±0.9	23.6±0.4	22.5±0.5	21.6±0.2
Day 18 BW (g)	51.6±1.2	50.6±2.0	53.4±2.4	53.0±0.6	49.7±2.2
Litter size (n)	11.5±0.7	10.7±0.8	10.7±0.8	11.0±0.9	9.4±0.7*
Foetal BW (g)	2.1±0.02	2.0±0.06	2.2±0.05	2.0±0.03*	1.9±0.03*
Foetal deaths (n)	0	0	1	2	2

Mice were injected intraperitoneally in two equally divided doses, once between 07.00 and 08.00 hours and again between 16.00 and 17.00 hours. Foetal body weight represents mean weight per litter. Day refers to day of gestation; BW, body weight. Dams were allowed to deliver normally and foetal body weight was recorded at 24 hours. * Different ($p < 0.05$) from the value for controls (0 TMA).

3.1.4.2 Organ weights, protein and DNA content

Administration of 2.5 mmol/kg/day TMA throughout gestation caused a decrease in the liver weights of 3 week-old male and female offspring and of brain, kidney and spleen weights of only male offspring. By 14 weeks of age only the seminal vesicle weight was significantly lower relative to controls.

When TMA was administered only during organogenesis, a dose of 5 mmol/kg/day caused a decrease in seminal vesicle weight and kidney weight of 8 week-old male offspring; no effect was apparent in female offspring. At 7.5 mmol/kg/day, there was a decrease in the weights of the kidney, brain, seminal vesicle and testes; kidney but not brain weights of female progeny were reduced (Table 6).

In 3 week-old offspring of mice treated with 7.5 mmol/kg/day TMA during organogenesis, total brain DNA (females only) and protein (males and females) was significantly less than corresponding controls. At 8 weeks of age a significant reduction in the amount of DNA and protein was recorded only in the male offspring (Table 6).

3.1.4.3 Plasma testosterone

Administration of 7.5 mmol/kg/day TMA during organogenesis did not cause a significant effect on serum testosterone levels of male offspring at 3 weeks of age; mean serum concentrations (nmol/litre) were 22 ± 17 in controls and 33 ± 27 in offspring of treated mice. However, at 8 weeks of age, testosterone levels in male offspring of TMA-treated mice were significantly lower than those in control offspring (Table 6).

Table 6. Effect of trimethylamine (TMA) injections (7.5 mmol/kg/day from day 6 to 15 of gestation) into mice on their progeny at 8 weeks of age

Variables	Male		Female	
	Control	TMA	Control	TMA
n	17	8	15	8
Body W (g)	42.3±0.7	38.2±.9*	30.1±0.5	27.7±0.6*
Brain W (mg)	526±10	495±10*	525±10	524±20
Liver W (mg)	2130±40	2060±100	1600±50	1510±60
Kidney W (mg)	330±11	297±15*	180±5	159±5*
Sem. Ves. W (mg)	221±10	179±10*	-	-
Serum T (nmol/L)	16.0±4.7	5.6±1.6*	not done	not done
Brain DNA (μg)	748±52	636±16*	600±11	668±13
Brain protein (mg)	34.9±0.5	32.4±0.5*	36.5±0.9	37.9±1.2

Pups were weaned and caged separately by sex at three weeks of age. Mice were killed at 8 weeks by cervical dislocation and organ weights recorded. Tissues were frozen at -80°C for later analysis. W, weight; Sem. Ves., seminal vesicle; T, testosterone. Brain DNA and protein represent mean total amounts. * Different ($p < 0.05$) from the value for the controls of the same sex.

3.1.4.4 Sexual functions

All female offspring from both control and TMA-treated (2.5 mmol/kg/day, day 1 to 17) mice exhibited regular oestrous cycling. The mean cycle length of control offspring (n=11) and of offspring (n=8) of TMA-treated mice was, respectively, 4.9 ± 0.2 and 5.1 ± 0.2 days.

There was no difference in the incidence or outcome of pregnancy resulting from mating of male and female offspring from treated mice with controls when compared to control females mated with control males. Eleven of 12 control females mated with control males, 10 out of 10 female offspring of TMA-treated mice mated with control males and 8 out of 10 control females mated with offspring of TMA-treated mice became pregnant within 5 days of cohabitation. There was no difference in the litter size or foetal body weight amongst these three groups.

3.1.5 General toxicity of S-(N-methylcarbamoyl)glutathione (SMG)

The injection of SMG at 100 mg/kg on day 12 of gestation was without any apparent effect on the dams. Weight gain in the treated groups occurred at the same rate as in controls and no differences were noted in foetal weights, placental weights, number of resorptions or foetal deaths. Pretreatment with buthionine sulfoxime also had no effect on the dams, with or without SMG at 100 or 500 mg/kg, when given on a single (day 12) or multiple days (days 10, 11, 12) in mid gestation (Table 7).

Table 7. Effect of buthionine sulfoxime (BSO) and S-(N-methylcarbamoyl)glutathione (SMG) on pregnancy development in mice

Variables	BSO	BSO + SMG
Dams (n)	5	6
Day 8 body weight (g)	32.7±0.9	32.5±0.9
Day 18 body weight (g)	51.8±3.2	52.2±2.0
Foetal body weight (g)	1.27±0.02	1.28±0.03
Placental weight (mg)	104±8	98±6
Foetal loss (%)	9.5±3.8	15.1±1.9

BSO (900 mg/kg) and SMG (100 mg/kg) were injected i.p. on days 10, 11 and 12 of gestation. SMG was administered 90 minutes after BSO.

3.2 *IN VITRO*

3.2.1 Effects of MIC on embryonic growth

Embryos removed from mice immediately after a 2 hour exposure to MIC on day 8 of gestation exhibited reduced growth and protein content when explanted and cultured *in vitro* for 42 hours (Table 8). No abnormalities were evident, either at the beginning or at the end of the culture period.

Injection of MIC vapour (714-2144 ppm) into the culture bottles at the beginning of culture resulted in death of all embryos at every concentration. Rat embryos cultured under similar conditions demonstrated concentration-dependent decreases in size and mortality (Varma *et al.*, 1990). Although MIC was added to the bottles in concentrations ranging from 714 to 2144 ppm, only 0-38 ppm could be detected 10 minutes after addition of MIC.

3.2.2 Effects of methylamines on embryonic growth

All three methylamines produced concentration-dependent decreases in yolk sac diameter, crown rump length and foetal survival (Figure 3). Developmental score and somite number also exhibited a similar concentration-dependent decrease. MMA was less toxic than DMA which was less toxic than TMA. The effect of all three methylamines was more marked on head length than on crown rump length or yolk sac diameter. Yolk sacs of embryos treated with DMA and TMA had a gross appearance of poor circulation. That is, they were pale and blood vessels were not conspicuous. Upon closer examination, however, the yolk sac was well vascularized but vessels were obscured by a cloudy yolk sac membrane. It was not determined whether the pale appearance was due to this obscuring effect or to reduced flow of blood in the vessels. The external appearance of embryos was not affected by low concentrations of methylamines. At higher concentrations (> 0.5 mM), there

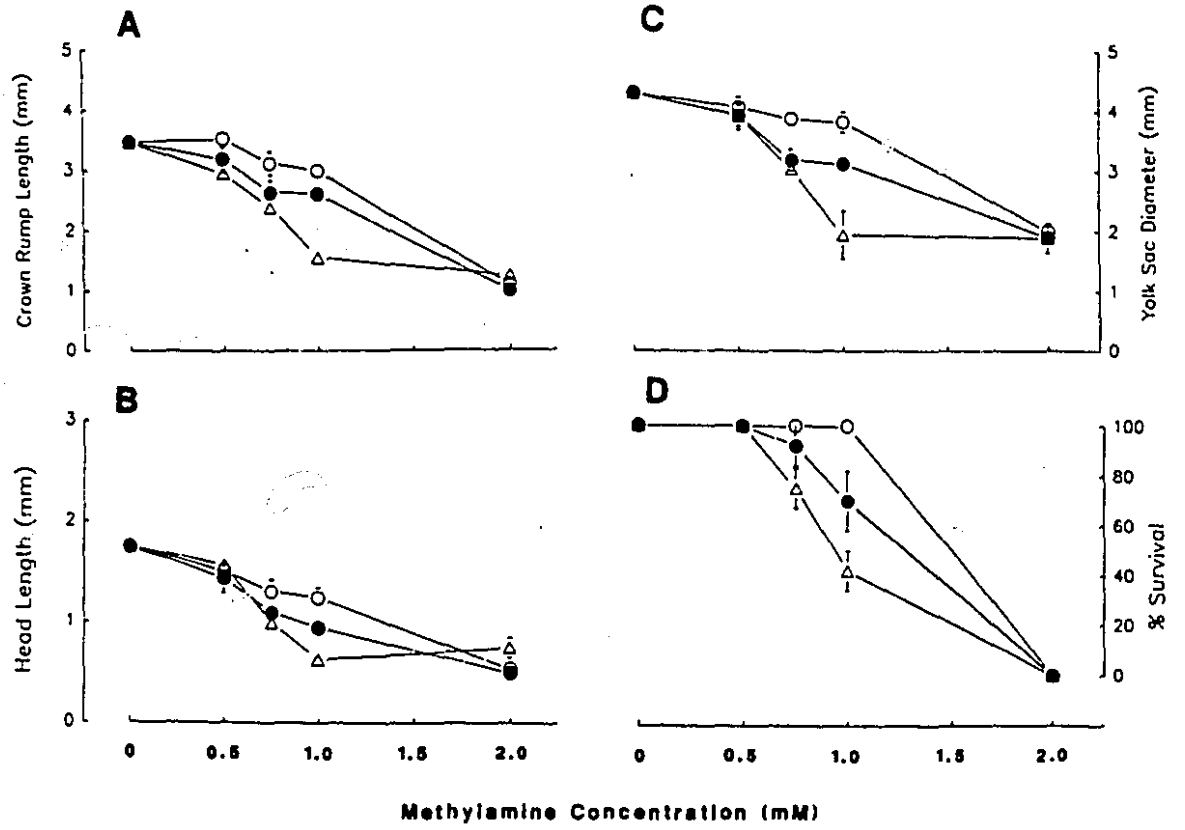

Figure 3. Effects of monomethylamine (o), dimethylamine (•) and trimethylamine (△) on (A) crown rump length, (B) head length, (C) yolk sac diameter and (D) survival of day 8 mouse embryos cultured 42 hours. Data represent the mean of 43 control embryos and 5-12 embryos in the presence of each concentration of methylamine. Vertical lines represent ± SEM. All measurements in the presence of methylamines were significantly ($p < 0.05$) different from controls at concentrations > 0.75 mM with the following exception: crown-rump length different at > 0.25 mM TMA.

Table 8. Effect of exposure of mice to MIC vapour on the development of their embryos *in vitro*

Variables	Control (air)	Methyl isocyanate (MIC)
Litters (n)	2	4
Embryos (n)	6	15
Yolk sac diameter (mm)	4.2±0.04	3.8±0.1*
Crown rump length (mm)	3.6±0.08	3.3±0.06*
Head length (mm)	1.9±0.09	1.7±0.1
Embryos dead (%)	0	0
Protein/embryo (μg)	78±7.5	43±8.5*

Mice were exposed to air or MIC (20 ppm) for 2 hours on day 8 of gestation, immediately killed by cervical dislocation and embryos removed and cultured under identical conditions.
* Different ($p < 0.05$) from the corresponding control.

appeared to be a disproportionate retardation in forelimb and branchial bar development relative to the development of other organs. All embryos were dorsally convex at 1 mM MMA or DMA, but this was true for only 33% of embryos at 1 mM TMA. The development of hearts was unaffected at concentrations up to 1 mM of all three methylamine and neuropores closed well up to concentrations of 0.75 mM.

All three methylamines produced concentration-dependent decreases in embryonic RNA, DNA and proteins; the relative order of toxicity was the same as *in vivo*, namely, TMA > DMA > MMA. (Figure 4). There appeared to be a relatively greater decrease in DNA than in RNA or proteins.

3.2.3 Characteristic effects of TMA on embryos

3.2.3.1 Embryonic growth

From the initial concentration-response studies, 0.75 mM TMA was chosen for all future experiments, unless otherwise noted. This concentration reduced growth and embryonic protein content to about 75% of control and led to mortality in approximately 30% of embryos. Details of growth parameters of embryos cultured in the presence of 0.75 mM TMA appear in Table 9. It is apparent that TMA is both teratogenic and embryotoxic.

TMA at 0.75 mM caused neural tube defects in 73% of embryos (Table 9). After a 42 hour exposure, 32% of embryos remained ventrally convex. The neural tube defects were most commonly characterized by a 'split head' appearance (a dorsal-rostral split) with formed but unfused neural folds. Treated embryos that achieved normal head morphology frequently had neural folds fused only at the level of somites 4/5; only occasionally was a formed (but open) posterior neuropore evident. Apart from the split head, embryos that had rotated demonstrated normal morphology.

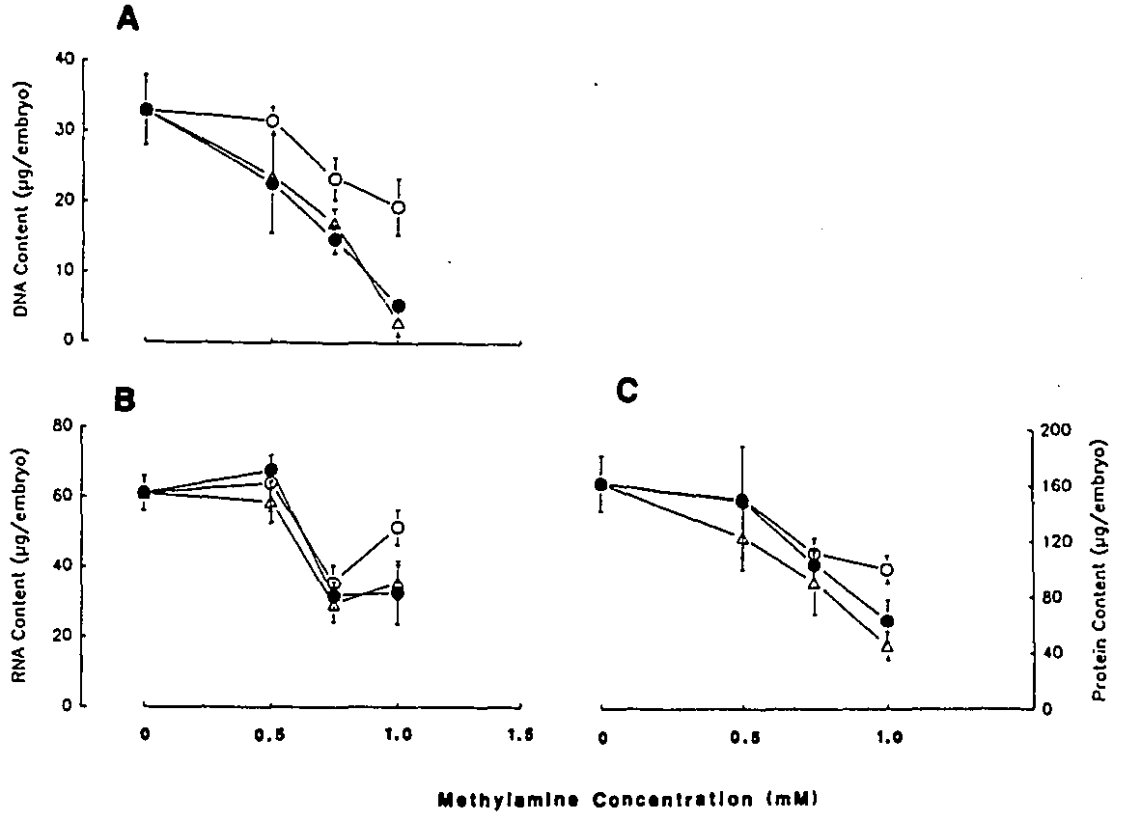

Figure 4. Effects of monomethylamine (○), dimethylamine (●) and trimethylamine (△) on (A) DNA, (B) RNA, and (C) protein contents of day 8 mouse embryos cultured 42 hours. DNA, RNA and proteins are significantly ($p < 0.05$) different from controls at 0.75-2 mM of all three methylamines with the following exceptions: protein is different at 0.5 mM TMA and DNA at 0.5 mM DMA and TMA. Data represent the mean of 8-12 control embryos and 4-10 embryos in the presence of each concentration of methylamine. Vertical lines represent ± SEM.

Table 9. Development of control and TMA-treated embryos after 42 hours of culture

Variables	Control	TMA-Treated
n	32	37
Yolk sac diameter (mm)	4.79±.10	2.99±.09*
Crown rump length (mm)	3.92±.05	2.43±.10*
Head length (mm)	1.87±.03	1.06±.03*
Developmental Score	35.6±.4	18.7±.8*
Somite (n)	34.8±.7	27.7±.8*
Embryos with NTD (n)	0	27*
Ventrally convex (n)	0	12*
Turning (n)	0	4*
Dorsally convex (n)	32	21*
Dead (n)	2	6*
Protein (µg/embryo)	213±9	131±14*
DNA Content (µg/embryo)	56±4	34±4*

Trimethylamine (0.75 mM) was added 1 hour after starting the cultures and all observations were made at 42 hours. NTD denotes neural tube defect. Units of all variables are numbers. * All variables in this column are significantly ($p < 0.05$) different from the corresponding values for control embryos.

Mortality in control embryos differed from that seen in treated embryos. Untreated embryos had developed fully and then died while TMA-treated embryos that were dead demonstrated poor or no development.

3.2.3.2 Macromolecular content and synthesis

DNA content of both untreated embryos and embryos treated with 0.75 mM TMA increased throughout the culture period (Figure 5). In the initial 16 hours of culture increase in DNA content of controls was not paralleled by increase in DNA in treated embryos. At 16, 24 and 42 hours the difference between controls and treated embryos became statistically significant. Between 24 and 42 hours, control embryos doubled their DNA content while TMA-treated embryos increased it by 70%.

Incorporation of ^{3}H-thymidine into DNA of control embryos increased steadily throughout culture, the rate doubling between 2 and 8 hours. In contrast, thymidine incorporation in treated embryos remained virtually unchanged for the first 16 hours of culture (Figure 5). At 4 hours this rate was 79% of control, steadily declining relative to control to be only 22% of control incorporation by 42 hours. There was a 4-fold increase in incorporation rate of controls between 24 and 42 hours and a 2.5-fold increase in treated embryos.

Despite a steady increase during the culture period, RNA content of treated embryos remained consistently lower than controls (Figure 6). By 2 hours, RNA content of treated embryos was only 75% of control, the highest level it ever achieved. As with DNA there was a doubling of RNA content of control embryos between 24 and 42 hours, while treated embryos exhibited a 70% increase.

The incorporation of ^{3}H-uridine into RNA steadily increased during the first 24 hours of

culture in both control and treated embryos (Figure 6). At 2 hours, the incorporation rate in treated embryos was 68% of the rate in controls. Unlike the incorporation of thymidine, the reduced incorporation rate of uridine into RNA of treated embryos achieved statistical significance by 4 hours.

The pattern of accumulation of protein in the two groups of embryos was similar to that shown by DNA and RNA, namely a reduced amount in treated embryos throughout the entire culture period (Figure 7). While protein content of controls doubled between 24 and 42 hours, only a 50% increase was observed in treated embryos in the same period.

The incorporation of ^{3}H-leucine into protein of TMA-treated embryos was 72% of control (its highest level) at 2 hours, steadily declining thereafter, relative to control, up until at least 24 hours (Figure 7).

3.2.3.3 Effects of thiol compounds and antioxidants

The results of culturing embryos in the presence of TMA and various antioxidants are listed in Table 10. Though L-cysteine addition produced marginally greater increases in size and development of TMA-treated embryos compared to D-cysteine, the difference between these stereoisomers was not significant (neither significantly increased growth, DNA or protein content in treated embryos). None of these agents had deleterious effects on embryonic development when added alone to cultures. A dose-response effect of L-cysteine on growth of TMA-treated embryos was observed but even at 1 mM L-cysteine growth of embryos was still < 80% of control. The frequency of neural tube defects was not altered by any concomitant treatment with these thiols or L-ascorbate.

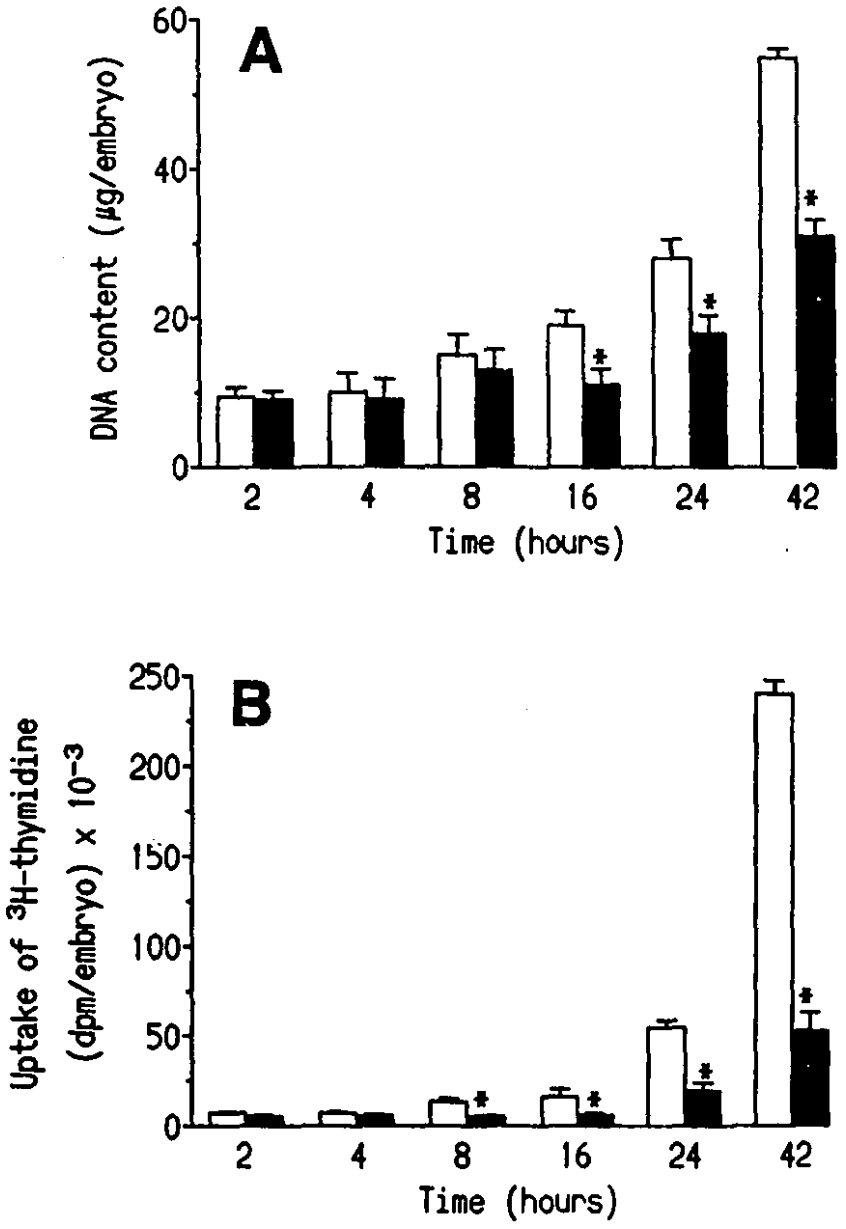

Figure 5. Effects of 0.75 mM trimethylamine (TMA) on (A) DNA content of and (B) incorporation of ^{3}H-thymidine by mouse embryos in culture as a function of time. TMA was present in the culture media from time 0 (1 hour after starting the culture) to the time of measurement and ^{3}H-thymidine (2µCi/ml) for a 2 hour period preceding the time indicated on the abscissa. White bars indicate absence and black bars indicate presence of TMA. Each mean value is derived from 4-6 separate embryos; vertical lines indicate ± SEM. * Denote significant ($p < 0.05$) difference from the corresponding control value.

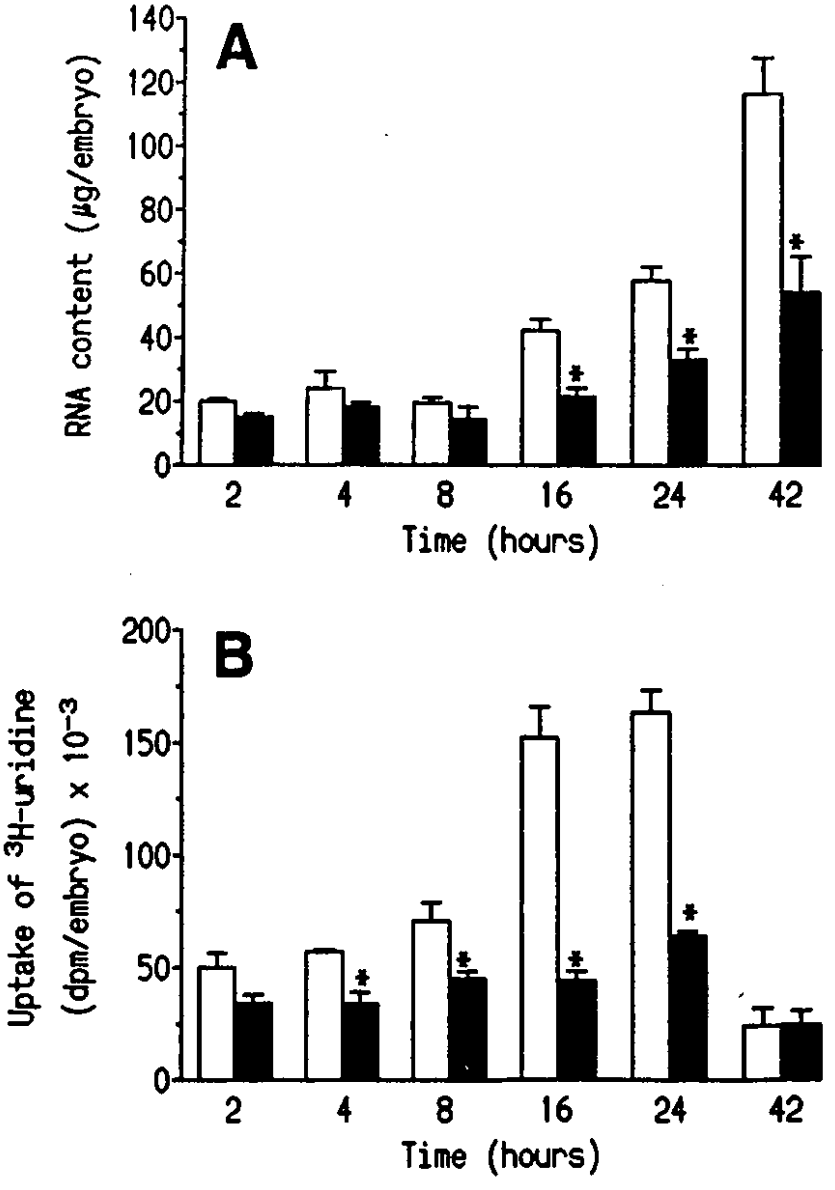

Figure 6. Effects of 0.75 mM trimethylamine (TMA) on (A) RNA content of and (B) incorporation of ^{3}H-uridine by mouse embryos in culture as a function of time. TMA was present in the culture medium from time 0 (1 hour after starting the culture) to the time of measurement and ^{3}H-uridine (2 µCi/ml) was present for 2 hours preceding the time shown on the abscissa. White bars indicate absence and black bars indicate presence of TMA. Each mean value is derived from 3-4 separate embryos; vertical lines indicate ± SEM. * Denote significant ($p < 0.05$) difference from the corresponding control value.

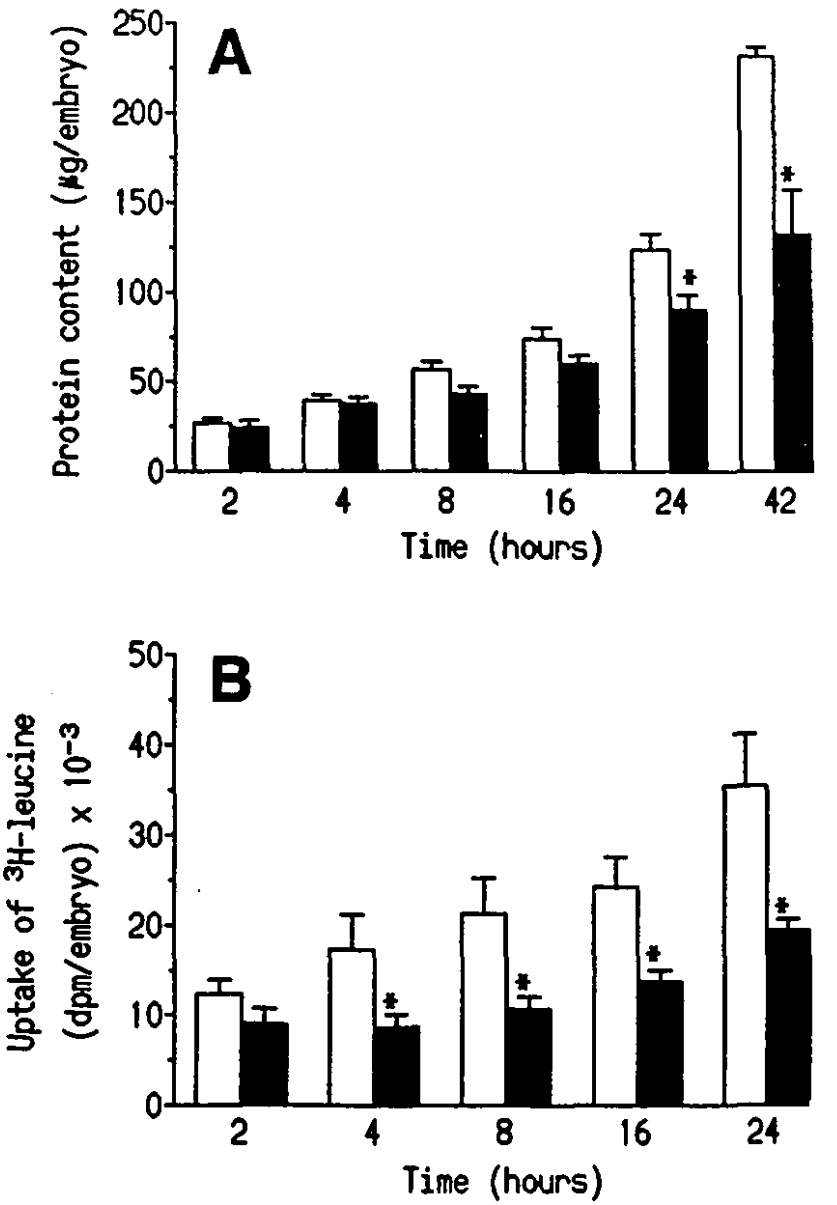

Figure 7. Effects of 0.75 mM trimethylamine (TMA) on (A) protein content of and (B) incorporation of ^{3}H-leucine by mouse embryos in culture as a function of time. TMA was present in the culture medium from time 0 (1 hour after starting the culture) to the time of measurement and ^{3}H-leucine (2 µCi/ml) was present for 2 hours preceding the time shown on the abscissa. White bars indicate absence and black bars indicate presence of TMA. Each mean value is derived from 5-8 separate embryos; vertical lines indicate ± SEM.
* Denote significant ($p < 0.05$) difference from the corresponding control value.

Table 10. Effects of L-cysteine (L-cys), D-cysteine (D-cys), glutathione (GSH) and ascorbic acid (Vit C) on the toxicity of trimethylamine (TMA) on mouse embryos in culture

Variables	Treatments					
	none	TMA	TMA + L-Cys	TMA + D-Cys	TMA + GSH	TMA + Vit C
n	19	19	18	15	11	9
YSD (mm)	4.5±0.1*	2.9±0.1	3.2±0.1	3.1±0.1	3.4±0.1	3.3±0.1
CRL (mm)	3.9±0.1*	2.4±0.1	2.6±0.2	2.5±0.2	2.7±0.1	2.7±0.2
HL (mm)	1.9±0.1*	1.1±0.1	1.2±0.1	1.1±0.1	1.1±0.1	1.1±0.1
DS	35±1*	19±1	20±2	17±2	24±1	22±1
Somite (n)	34±1*	28±1	29±1	27±2	33±1	30±1
Protein (μg/E)	213±9*	131±14	141±17	113±18	160±21	94±14
DNA (μg/E)	56±4*	34±4	38±3	31±7	41±7	44±10

Thiols and ascorbic acid (100 μM) were added to the culture medium 30 minutes and TMA 60 minutes after the beginning of culture and scoring was done at 42 hours. YSD, yolk sac diameter; CRL, crown rump length; HL, head length; DS, developmental score; E, embryo. * Different ($p < 0.05$) from all other values in the same row with the exception that somite number in control and TMA + GSH groups and DNA content in control and TMA + Vit C groups do not differ from each other.

3.2.3.4 Effects of growth factors

The presence of insulin or vitamin A in the culture media produced little effect on the growth of embryos (Figure 8). The addition of IGF-II significantly increased embryonic protein but other parameters of growth, including yolk sac diameter, crown rump length and number of somites were not increased. The addition of zinc led to reduced yolk sac diameter, crown rump length and embryonic protein (Figure 8). In the presence of 1 mM TMA, none of the growth factors were able to normalize growth, although IGF-II and zinc significantly increased embryonic protein content in comparison to TMA treatment alone (Figure 8).

3.2.4 Characteristic effects of SMG

3.2.4.1 Embryonic growth

SMG inhibited the growth and development of embryos in culture in a concentration-dependent manner (Figure 9). Effects of SMG were apparent at a concentration of 0.1 mM and complete suppression of embryonic growth occurred at 2 mM. At 0.25 mM, SMG reduced yolk sac and embryonic size to approximately 75% of control and the embryonic protein content to 63% of control and this concentration of SMG was used in all subsequent experiments. In addition to growth retardation, a significant number of embryos failed to rotate; the incidence of this defect after 42 hours of culture was 18% at 0.25 mM SMG, 60% at 0.5 mM and 100% at 0.75 mM. Despite this severe reduction in development, SMG did not cause a significant increase in embryo mortality as judged by a beating heart, which was absent in only 1 out of 9 embryos treated with 2 mM SMG as compared with 2 out of 17 control embryos.

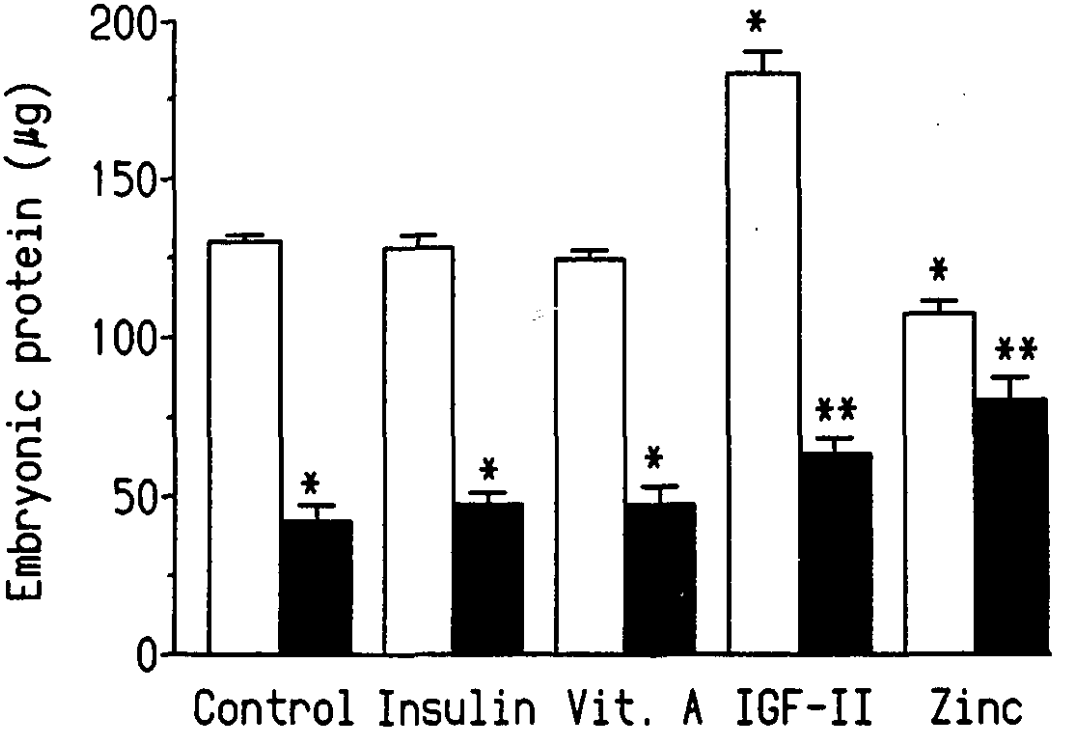

Figure 8. Effect of trimethylamine (TMA) and growth factors on mouse embryonic protein content. Day 8 embryos were cultured for 42 hours in the absence (white bars) or presence (black bars) of 1 mM TMA. Insulin (5 nM), IGF-II (100 ng/ml), vitamin A (0.1 µg/ml) and zinc (10 µg/ml) were added at the beginning of culture, 30 minutes before the addition of TMA. Data are means of 4-6 separate embryos from separate litters. Vertical lines represent ± SEM. * Denote significant ($p<0.05$) differences compared to untreated; ** denote significant ($p<0.05$) differences compared to TMA alone.

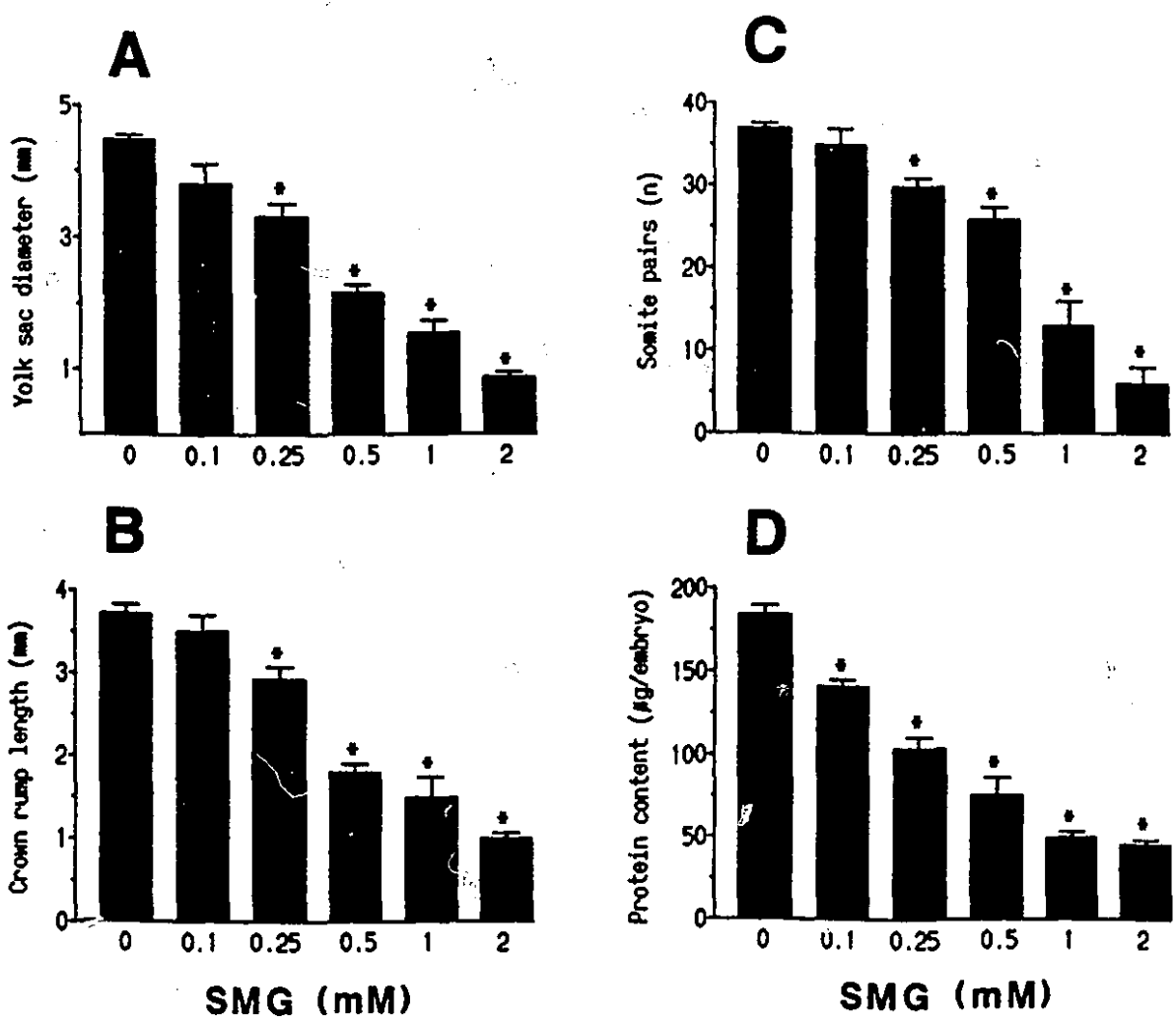

Figure 9. Effects of S-(N-methylcarbamoyl)glutathione (SMG) on growth of embryos *in vitro*. Embryos were explanted on day 8 and cultured for 42 hours at which time yolk sac diameter (A), crown rump length (B), somite number (C) and embryonic protein content (D) were determined. White bars indicate absence and black bars indicate presence of SMG. Vertical lines represent ± SEM of 8-10 separate embryos from separate litters. All values in the presence of SMG are significantly ($p < 0.05$) different from control values with the exception of yolk sac diameter, crown rump length and somite number at 0.1 mM SMG.

Those embryos that did rotate in the presence of 0.25 mM SMG appeared morphologically normal except for a characteristic spinal abnormality. Six of 16 treated embryos demonstrated spinal axial twists and/or kinks; there was irregular spacing and separation of individual somites and of somite pairs and divergence of somite pairs from the normal spinal axis. The presence of this abnormality was not limited to dorsally convex embryos but at concentrations of SMG $\geq$ 1.0 mM it was not easily confirmed due to poor development.

3.2.4.2 Macromolecular content and synthesis

There was a time-dependent increase in embryonic and yolk sac DNA in SMG-treated embryo cultures. For the first 4 hours, DNA content was significantly greater in SMG-treated embryos and it remained higher than control at 8 hours (Figure 10A). However, by 16 hours control embryos had significantly more DNA and this remained so at 24 and 42 hours. At the end of culture DNA content of SMG-treated embryos was 65% of control.

DNA content of treated yolk sacs was also greater than control during the first 8 hours but significantly so only at 8 hours (Figure 11A). By 24 hours control yolk sacs contained more DNA and by 42 hours, when treated yolk sacs had a DNA content that was 56% of the control level, this difference was significant.

The incorporation of ^{3}H-thymidine into embryonic DNA steadily increased throughout the culture in SMG-treated embryos; the maximum increase occurred between 8 and 16 hours (Figure 10B). However, the incorporation of thymidine was significantly less in SMG-treated embryos than in control embryos from 16 to 42 hours of culture.

The incorporation of ^{3}H-thymidine into control yolk sacs was similar to that into control embryos; there was a steady increase throughout the culture period (Figure 11B). In

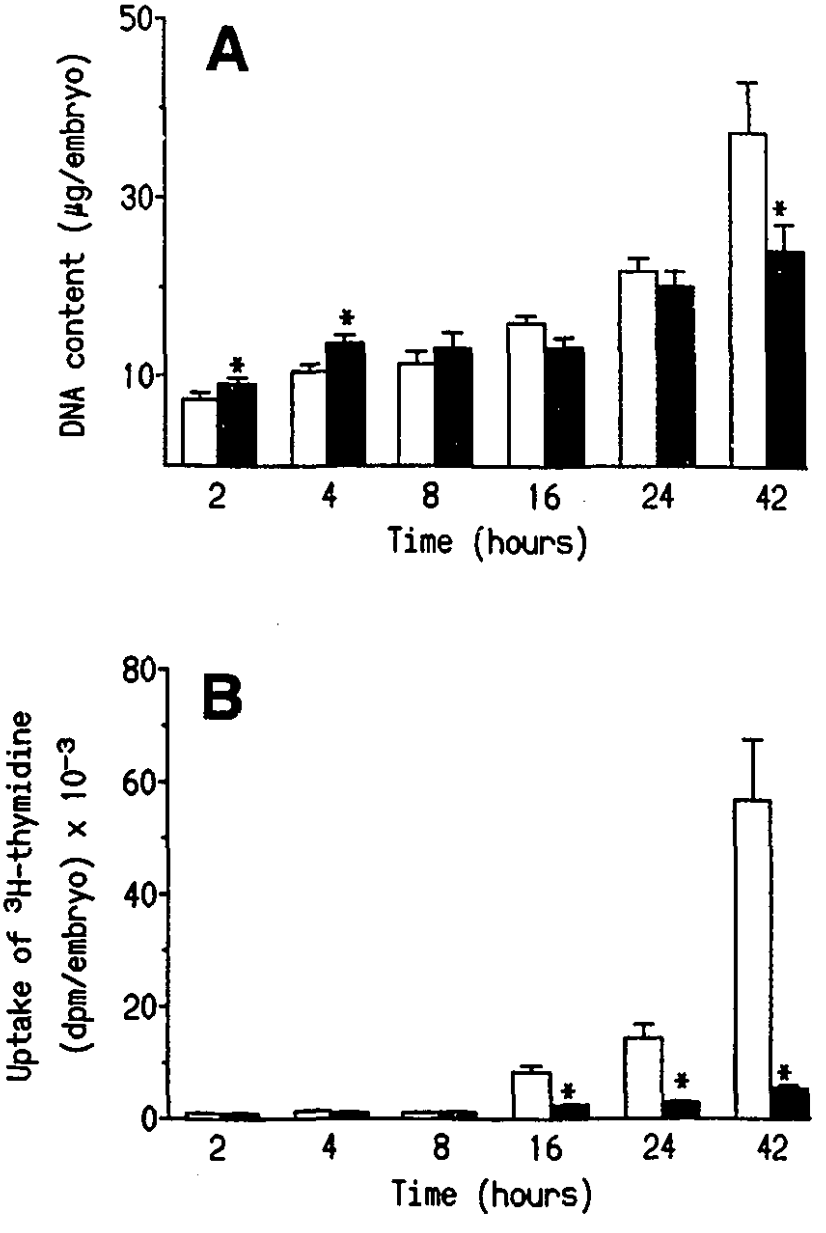

Figure 10. Effects of 0.25 mM S-(N-methylcarbamoyl)glutathione (SMG) on DNA content (A) of and ^{3}H-thymidine incorporation (B) by day 8 mouse embryos cultured for the indicated time-periods. ^{3}H-Thymidine (2 µCi/ml) was present for 2 hours preceding the time shown on the abscissa. White bars indicate absence and black bars indicate presence of SMG. Vertical lines represent ± SEM of 4-6 separate embryos from separate dams. * Denote significant ($p < 0.05$) difference from the corresponding control value.

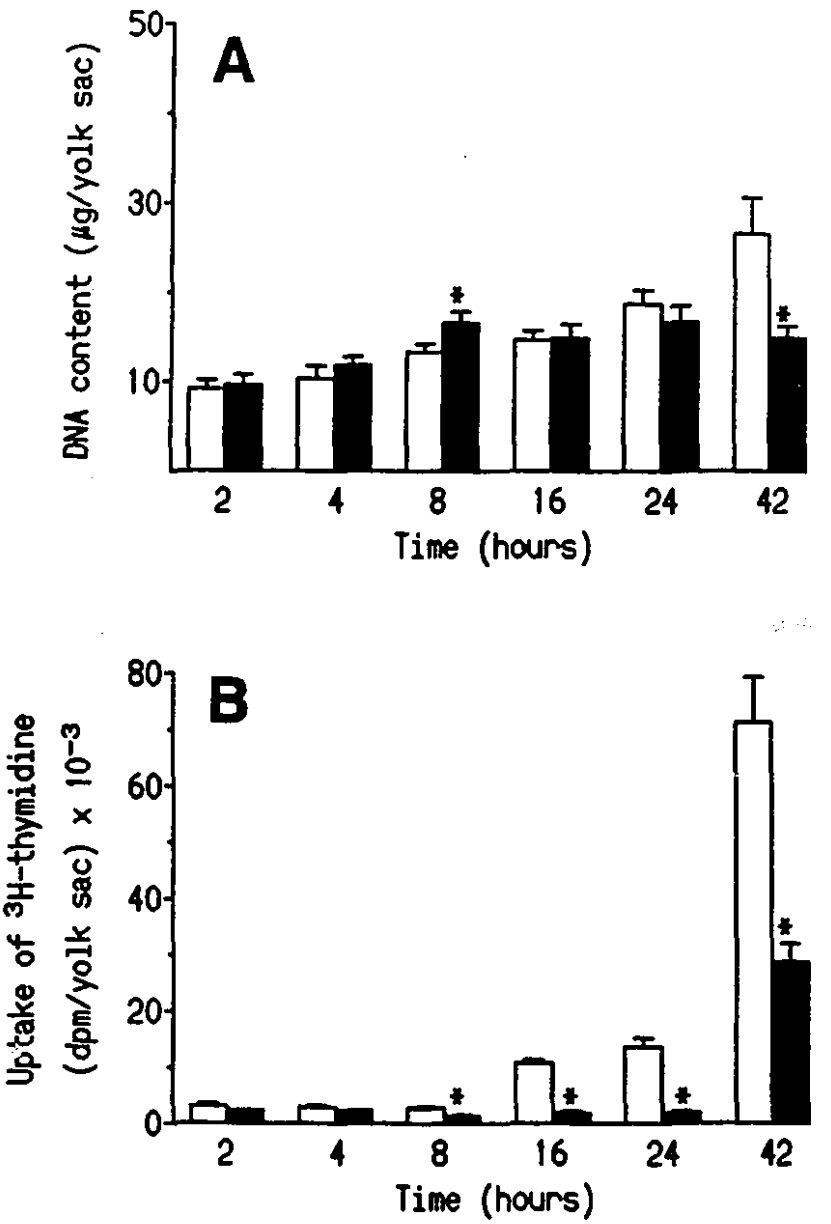

Figure 11 . Effects of 0.25 mM S-(N-methylcarbamoyl)glutathione (SMG) on DNA content (A) of and ^{3}H-thymidine incorporation (B) by yolk sacs of day 8 embryos cultured for the indicated time-periods. ^{3}H-Thymidine (2 µCi/ml) was present for 2 hours preceding the time shown on the abscissa. White bars indicate absence and black bars indicate presence of SMG. Vertical lines represent ± SEM of 4-6 separate embryos from separate dams. * Denote significant ($p < 0.05$) difference from the corresponding control value.

contrast, ^{3}H-thymidine incorporation into yolk sacs of SMG-treated embryos remained virtually unchanged for the first 24 hours and an increase was observed only in the last 18 hours of culture. The incorporation of thymidine by yolk sacs of SMG-treated embryos was significantly less than that by yolk sacs of control embryos from 8 to 42 hours of culture.

At all concentrations of SMG (0.1-2 mM), inhibition in morphologic growth was accompanied by a significant decrease in embryonic and yolk sac protein content (Figure 12). The time-course of decrease in protein concentration was determined with 0.25 mM SMG; a significant reduction in embryonic and yolk sac protein content was observed at 16-42 hours of culture (Figure 12). At 24 hours, embryonic protein (μg/embryo) was 70 ± 6 in control and 49 ± 6 in SMG-treated embryos and yolk sac protein (μg/yolk sac) was 97 ± 6 in control and 76 ± 6 in treated embryos.

3.2.4.3 Effects of thiol compounds

Data on the effects of the addition of (-)-2-oxo-4-thiazolidine-carboxylic acid (OTC) and glutathione (GSH), with and without SMG, are presented in Table 11. At 3 mM, GSH inhibited SMG embryotoxicity. L-Cysteine at 0.1 mM and OTC at 4 mM did not inhibit SMG's embryotoxic effects on morphologic development and protein content but did reduce spinal kinking. Effects of higher concentrations of L-cysteine and OTC on SMG embryotoxicity were not studied because L-cysteine at 1 mM and OTC at 6 mM was toxic to embryos.

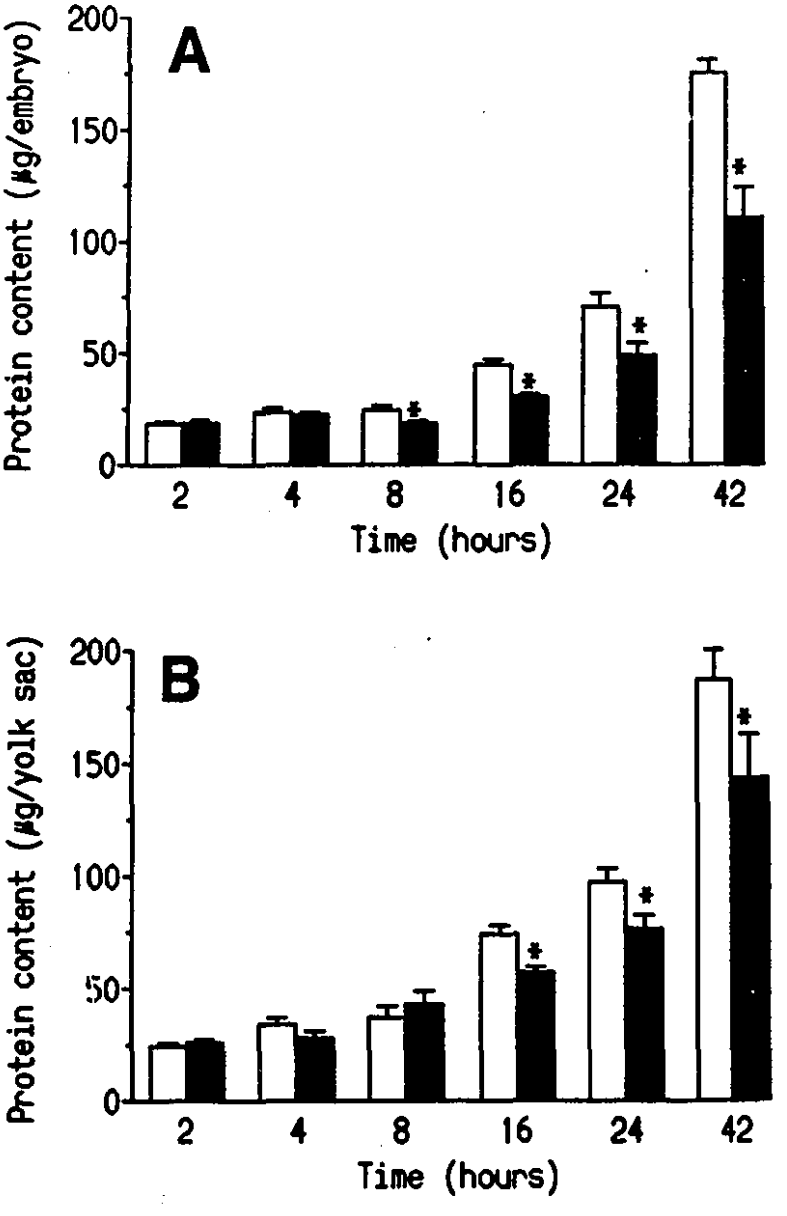

Figure 12. Embryonic (A) and yolk sac (B) protein content of day 8 embryos cultured with 0.25 mM S-(N-methylcarbamoyl)glutathione (SMG). SMG was added 30 minutes after the start of culture. White bars indicate absence and black bars indicate presence of SMG. Vertical lines represent ± SEM of 3-7 embryos from separate litters. * Denote significant ($p < 0.05$) difference from the corresponding control.

Table 11. Modification by thiol donors of SMG toxicity on mouse embryos in culture

Treatment	n	YS (mm)	CR (mm)	Somite (n)	Spinal defects	Protein (μg/YS)	Protein (μg/embryo)
None	17	4.4±0.1	3.7±0.1	36±1	0	185±7	155±4
SMG	16	3.3±0.2*	2.9±0.2*	30±1*	6*	163±8*	96±5*
GSH (1 mM)	7	4.3±0.2	3.6±0.1	35±1	0	175±6	187±12*
GSH (1 mM) + SMG	6	3.4±0.1*	3.1±0.1*	33±1	1	165±9*	125±12*
GSH (3 mM)	8	4.5±0.2	3.4±0.1	34±1	0	207±7*	150±5
GSH (3 mM) + SMG	7	4.3±0.1	3.4±0.1	34±1	0	198±9	120±6*
OTC (4 mM)	7	5.4±0.1*	4.3±0.2*	37±1	0	192±10	166±8
OTC (4 mM) + SMG	7	3.4±0.1*	3.0±0.1*	31±1*	2	110±5*	82±6*

Treatment (SMG, 0.25 mM) was begun 1 hour after starting the cultures and measurements were made at 42 hours. SMG, S-(N-methylcarbamoyl)GSH; CYS, L-cysteine; GSH, glutathione; OTC, (-)-2-oxo-4-thiazolidine-carboxylic acid; YS, yolk sac; CR, crown rump length. Concentration of SMG was 0.25 mM. * Significantly ($p < 0.05$) different (ANOVA) (except for spinal defects, which were analyzed by the Chi-square test) from the value for the untreated group in the same column.

3.2.5 Effects on yolk sacs in culture

3.2.5.1 Effects of TMA on uptake of ^{14}C-sucrose and ^{125}I-BSA

There was a steady uptake of ^{14}C-sucrose by yolk sacs of day 9 embryos during 5 hours of culture; in isolated day 15 yolk sacs, uptake was linear over the first 4 hours only (Figures 13 and 14). The capacity for uptake of ^{14}C-sucrose by day 9 embryos was approximately twice that of day 15 yolk sacs. TMA treatment did not result in a significant change in uptake by either tissue, with the following exception: uptake at 1 hour in TMA-treated embryos was significantly less than uptake in corresponding controls (Figures 13 and 14).

There was a steady uptake of ^{125}I-BSA by isolated yolk sacs throughout 5 hours of culture (Figure 15). Inhibition of ^{125}I-BSA uptake by TMA was significant by 3 hours and remained so at 5 hours. At this time, uptake in TMA-treated yolk sacs was 75% of control. Inhibition of uptake by 5 mM TMA was completely reversible upon removal of TMA (Figure 15). Leupeptin increased uptake of ^{125}I-BSA (Table 12).

3.2.5.2 Effects of TMA on lysosomal degradation of ^{125}I-BSA

When isolated day 15 yolk sacs were cultured in the presence of ^{125}I-BSA, there was a steady accumulation of TCA-soluble radioactivity in the culture medium. In the presence of TMA or leupeptin, there was a significant reduction in the amount of TCA-soluble counts which appeared in the media (Table 12). When the data were expressed as the ratio of TCA-soluble counts to uptake of ^{125}I-BSA, there was no difference between control and TMA-treated yolk sacs but this ratio was significantly reduced in the presence of leupeptin. Concentration-response curves revealed that lysosomal degradation of ^{125}I-BSA independent of uptake was not significantly inhibited until the concentration of TMA reached 5 mM (Figure 16).

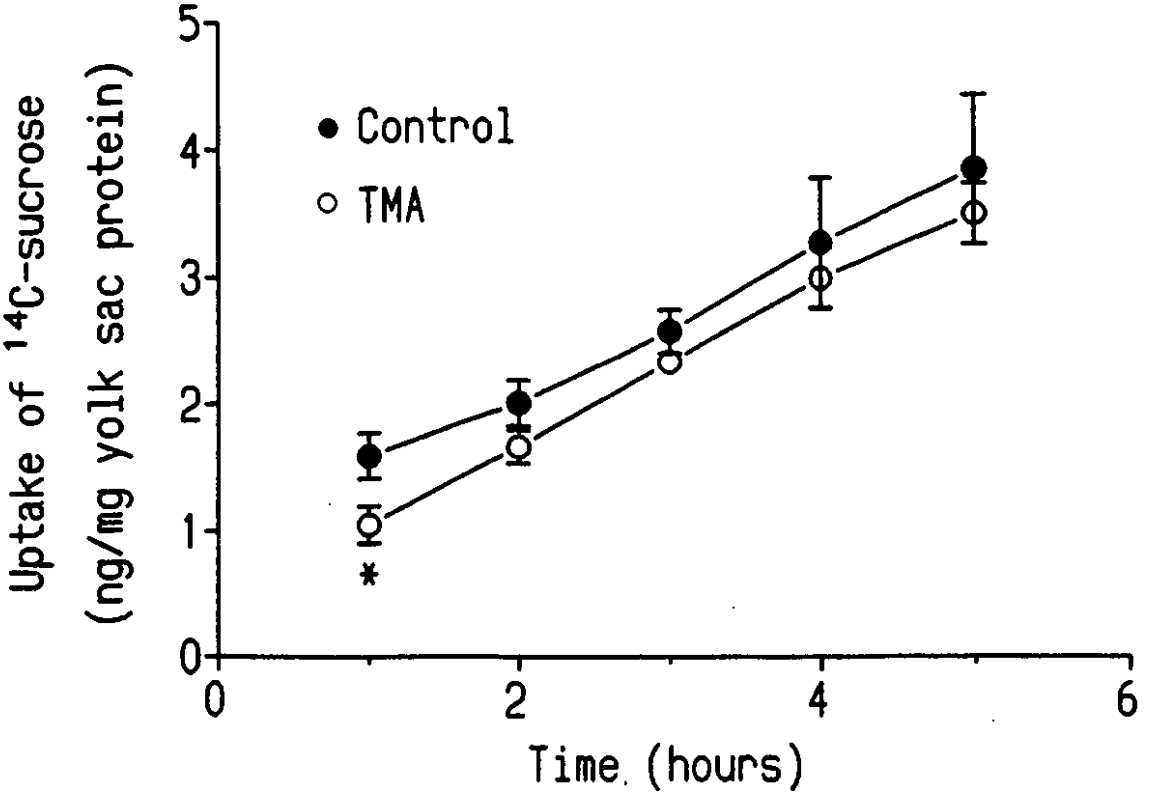

Figure 13. Effect of trimethylamine (TMA) on ^{14}C-sucrose uptake by yolk sacs of day 9 mouse embryos. Embryos were explanted on day 8, cultured overnight and ^{14}C-sucrose (1 µCi/ml) was added the following morning for a 5 hour labelling period. Data are means of 5-7 separate embryos from separate litters. Vertical lines represent ± SEM. * Denote significant ($p < 0.05$) difference from the corresponding control.

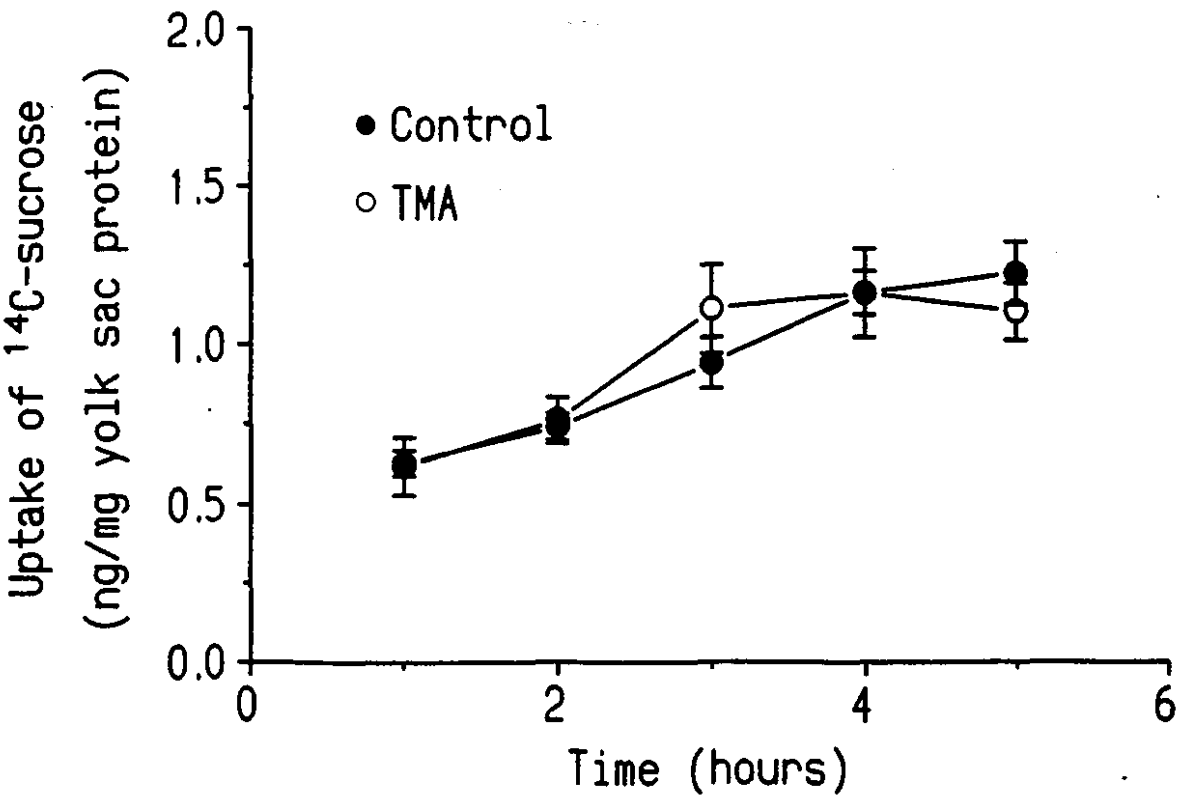

Figure 14. Effect of trimethylamine (TMA) on ^{14}C-sucrose uptake by day 15 isolated mouse yolk sacs. Embryos were explanted on day 15 and the yolk sacs removed and cultured individually. ^{14}C-Sucrose (1 μCi) was added after 30 minutes preincubation with TMA. Data are means of 12-15 separate experiments. Vertical lines represent ± SEM.

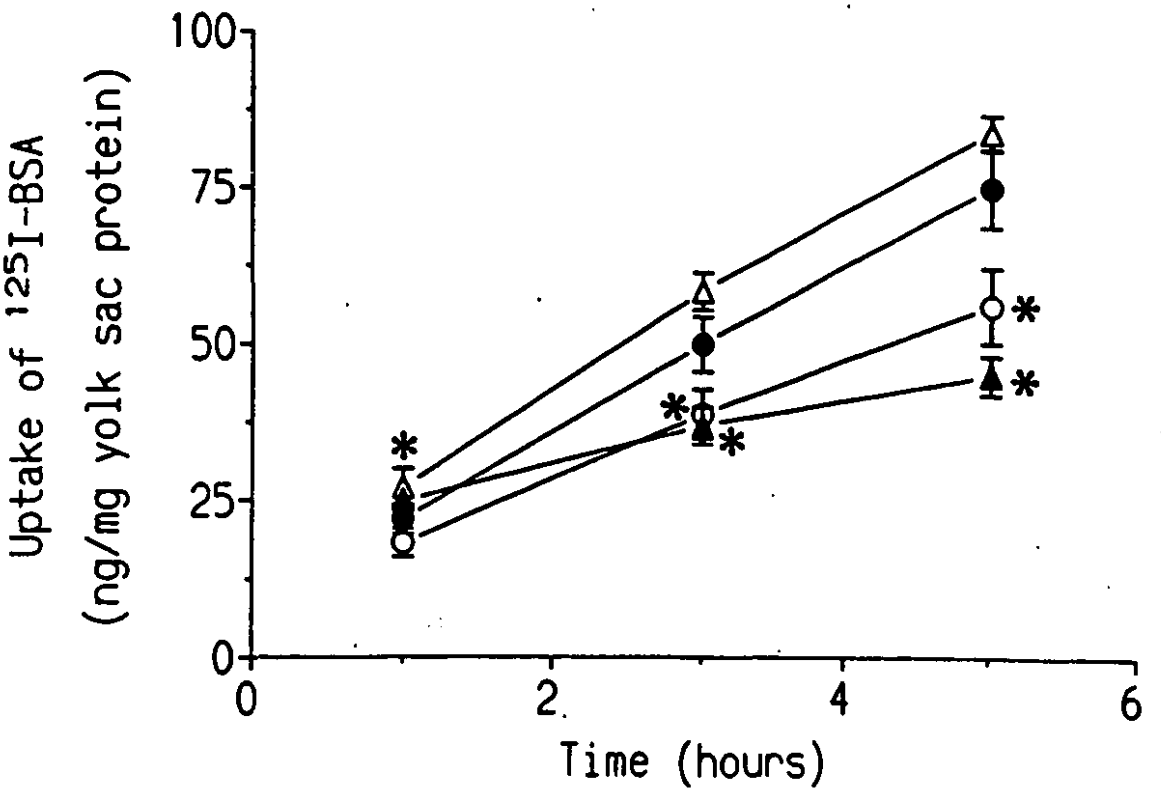

Figure 15. Uptake of ^{125}I-bovine serum albumin (BSA) in isolated day 15 mouse yolk sacs. Embryos were explanted on day 15 and the yolk sacs removed and cultured individually. Yolk sacs were preincubated for 30 min without (●) or with trimethylamine (TMA) at 0.75 mM (○) or 5 mM (▲) or for 1 hr with TMA at 5 mM, followed by rinsing and subsequent culturing without TMA (△). ^{125}I-BSA (0.1 µCi/ml) was added at the end of the preincubation period. Data are means of 3-5 separate yolk sacs from separate litters. Vertical lines represent ± SEM * Denote significant ($p < 0.05$) difference from corresponding controls.

Table 12. Effect of trimethylamine (TMA) on the uptake and lysosomal degradation of ^{125}I-BSA (bovine serum albumin) by isolated day 15 mouse yolk sac (YS)

Variables	Control	TMA (0.75 mM)	Leupeptin (53 μM)
Uptake of ^{125}I-BSA (ng/mg YS protein)	64.91±2.3	52.44±2.4*	74.1±1.5*
TCA-soluble dpm/ mg YS protein	126,483±4,530	105,198±6,091*	106,725±7,250*
TCA-soluble dpm/ ng ^{125}I-BSA taken up	1,187±29	1,198±46	873±44*
Protein/YS (μg)	1,648±73	1,640±72	1,728±116

Individual yolk sacs were cultured for 5 hours in 10 ml of medium 199 containing 0.1 μCi/ml ^{125}I-BSA. Data represent values at 5 hours and are derived from 10-12 separate yolk sacs from 10-12 separate dams. Yolk sacs were incubated untreated for 30 minutes followed by 30 minutes in the presence of TMA; ^{125}I-BSA was then added. * Different ($p < 0.05$) from the corresponding control value.

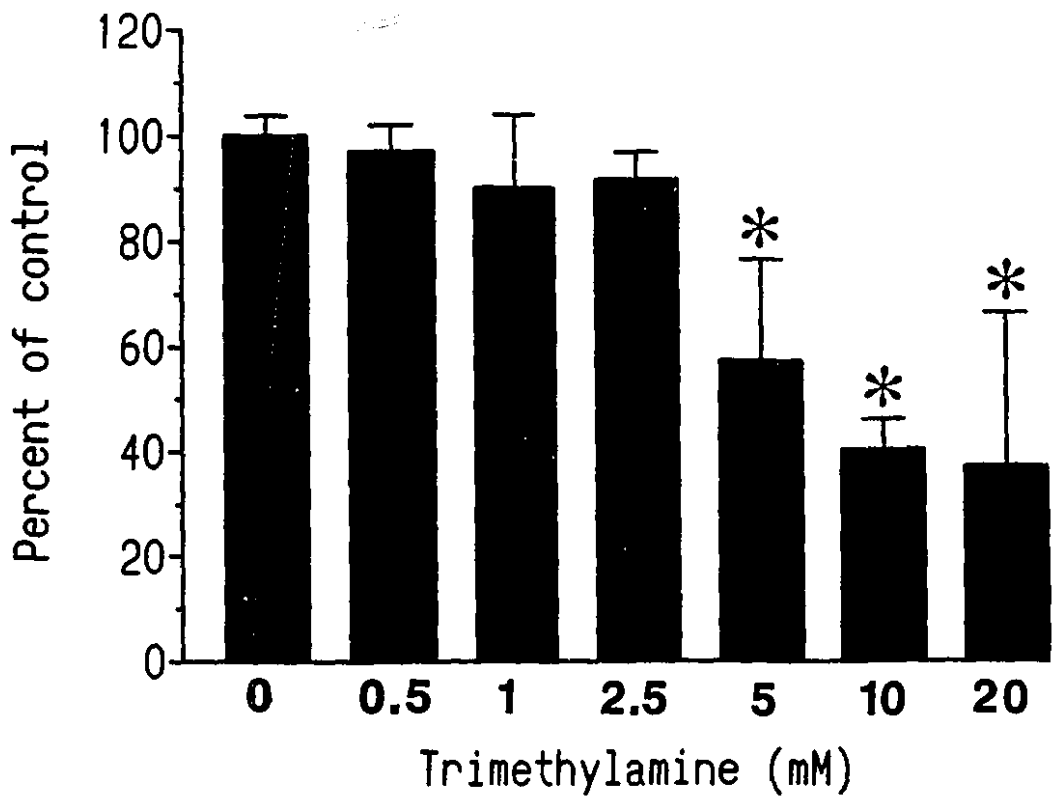

Figure 16. Ratio of trichloroacetic acid-soluble dpm to uptake of ^{125}I-bovine serum albumin (BSA) as a function of trimethylamine (TMA) concentration. Individual day 15 mouse yolk sacs were preincubated for 30 minutes before addition of ^{125}I-BSA. Data represent values at the end of 5 hours and are the means of 3-4 separate yolk sacs from separate litters. Vertical lines represent ± SEM. * Denote significant ($p < 0.05$) difference from corresponding controls.

3.2.5.3 Effects of SMG on uptake of ^{14}C-sucrose and ^{125}I-BSA

SMG caused a significant inhibition of ^{14}C-sucrose uptake by isolated day 15 yolk sacs within 1 hour and the uptake was 62% of the control value at 5 hours (Figure 17). SMG also significantly inhibited uptake of ^{125}I-BSA uptake which was significant at 5 hours and represented 77% of uptake in untreated yolk sacs (Table 13 and Figure 18).

Concentrations of N-acetylcysteine (NAC) and glutathione (GSH) up to 1 mM did not exert a significant effect on the uptake of ^{125}I-BSA by day 15 yolk sacs but both these agents inhibited BSA uptake at 2 mM (Figures 19 and 20). SMG caused a significant inhibition of ^{125}I-BSA uptake in the absence but not in the presence of 1 mM GSH. Lower concentrations of GSH or concentrations of NAC up to 2 mM did not significantly antagonize SMG-induced inhibition of ^{125}I-BSA uptake.

In concentrations up to 1 and 0.25 mM, respectively, acivicin and aminooxyacetic acid did not exert a significant effect on the uptake of ^{125}I-BSA by day 15 yolk sacs (Figures 21 and 22). Higher concentrations inhibited uptake of ^{125}I-BSA. Neither agent was able to antagonize the inhibitory effects of 0.25 mM SMG at any concentration up to 2 mM.

3.2.5.4 Effects of SMG on lysosomal degradation of ^{125}I-BSA

When isolated day 15 yolk sacs were cultured with SMG in the presence of ^{125}I-BSA, there was a significant reduction in the amount of TCA-soluble counts which appeared in the media (Table 13). When the data was expressed as the ratio of TCA-soluble counts to uptake of ^{125}I-BSA, there was no difference between control and SMG-treated yolk sacs.

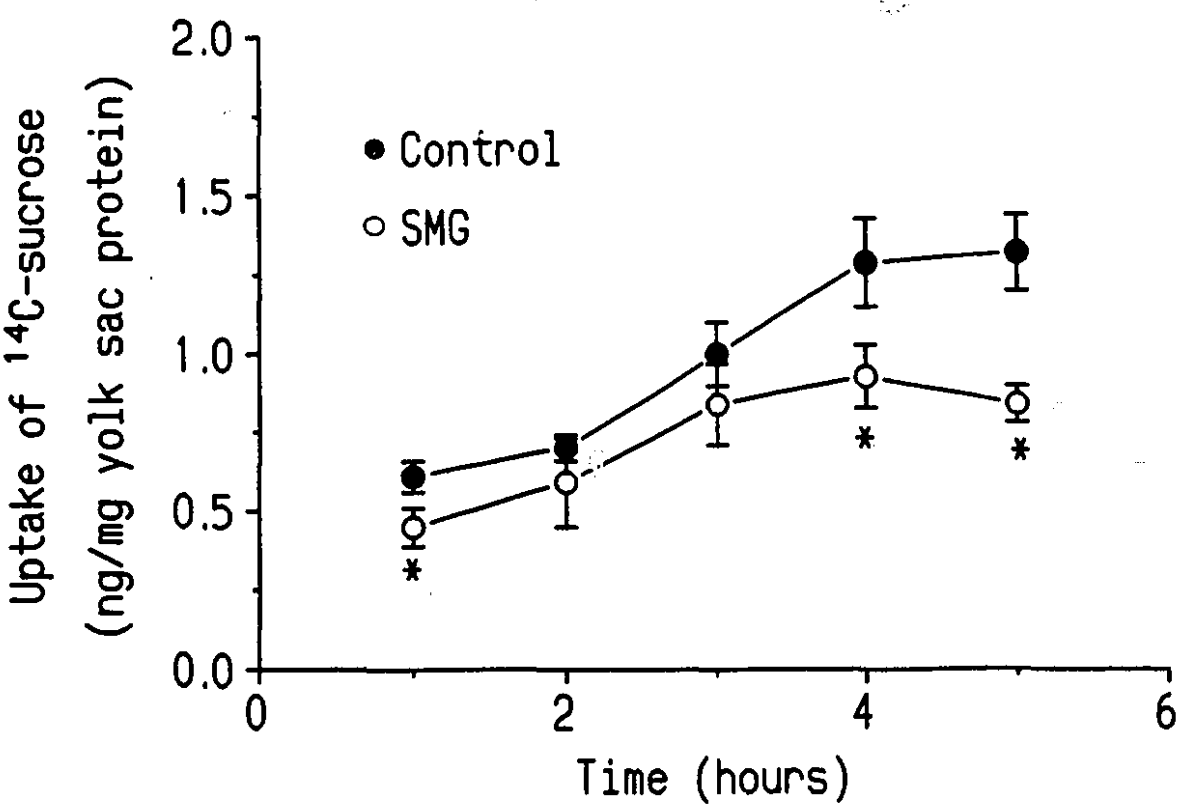

Figure 17. Effect of S-(N-methylcarbamoyl)glutathione (SMG) on ^{14}C-sucrose uptake by day 15 isolated mouse yolk sacs. Embryos were explanted on day 15 and the yolk sacs removed and cultured individually. ^{14}C-Sucrose (1 μCi) was added after 30 minutes preincubation with SMG. Data are means of 5-7 separate yolk sacs from separate litters. Vertical lines represent ± SEM. * Denotes significant ($p < 0.05$) difference from the corresponding control value.

Table 13. Effect of S-(N-methylcarbamoyl)glutathione (SMG) on the uptake and lysosomal degradation of ^{125}I-BSA (bovine serum albumin) by isolated day 15 mouse yolk sacs (YS)

Variables	Control	SMG (0.25 mM)
Uptake of ^{125}I-BSA (ng/mg YS protein)	75.2±6.3	58.1±2.7*
TCA-soluble dpm/mg YS protein	125,216±10,911	103,964±4,989*
TCA-soluble dpm/ng ^{125}I-BSA taken up	1,027±80	1,047±134
Protein/YS (μg)	1,576±64	1,538±84

Individual yolk sacs were cultured for 5 hours in 10 ml of medium 199 containing 0.1 μCi/ml ^{125}I-BSA. Data represent values at 5 hours and are the means of 10-12 separate yolk sacs from 10-12 separate dams. Yolk sacs were incubated untreated for 30 minutes followed by 30 minutes in the presence of SMG; ^{125}I-BSA was then added. * Different ($p < 0.05$) from the corresponding control value.

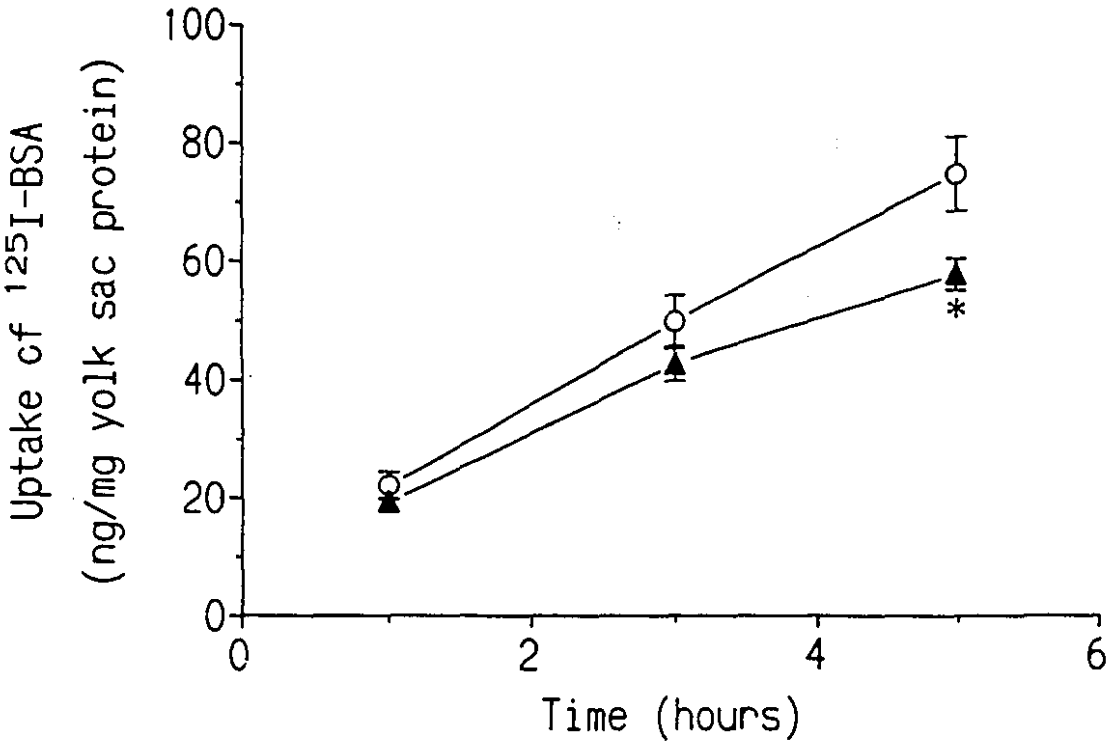

Figure 18. Uptake of ^{125}I-bovine serum albumin (BSA) in isolated day 15 mouse yolk sacs. Embryos were explanted on day 15 and the yolk sacs removed and cultured individually. Yolk sacs were preincubated for 30 minutes without (o) or with (▲) S-(N-methylcarbamoyl)-glutathione (SMG) at 0.25 mM. ^{125}I-BSA (0.1 μCi/ml) was added at the end of the preincubation period. Data are means of 12-15 separate yolk sacs from separate litters. Vertical lines represent ± SEM. * Denotes significant ($p < 0.05$) difference from the corresponding control.

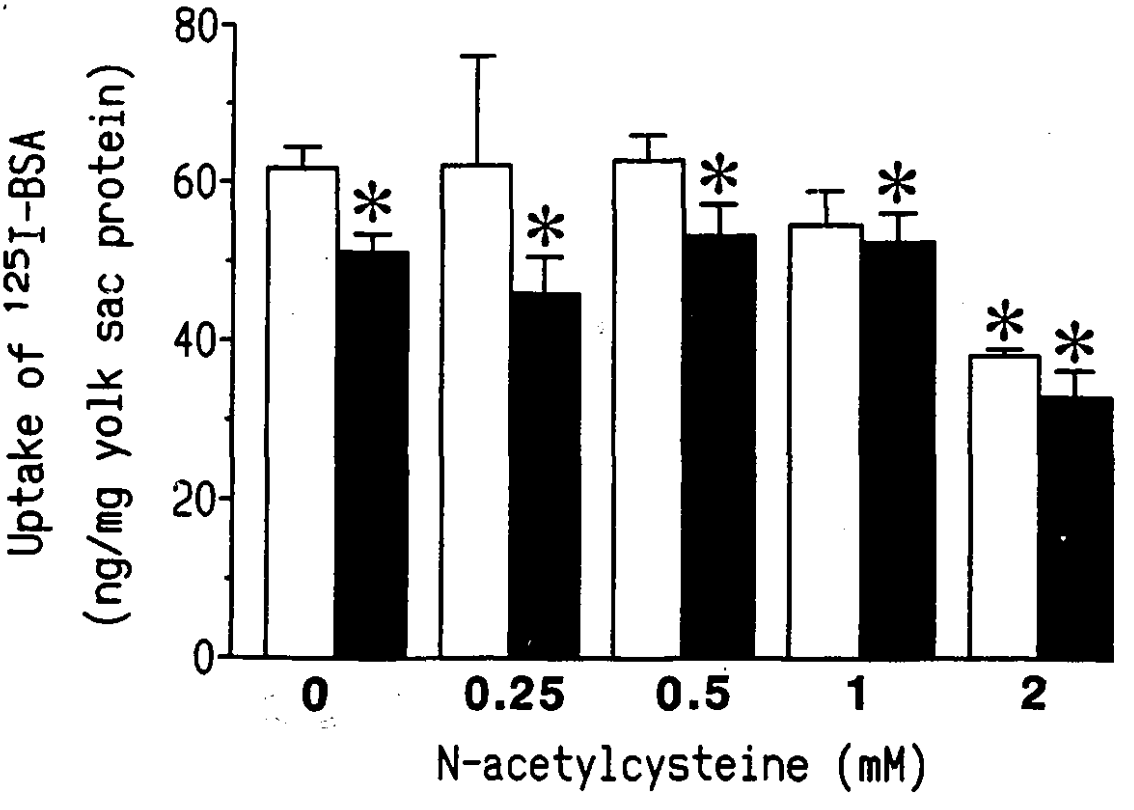

Figure 19. Effect of S-(N-methylcarbamoyl)glutathione (SMG) and N-acetylcysteine (NAC) on the uptake of ^{125}I-BSA (bovine serum albumin) by isolated day 15 mouse yolk sacs. Yolk sacs were cultured individually in 10 ml medium 199. NAC was added 30 minutes before and ^{125}I-BSA (0.1 µCi/ml) 30 minutes after SMG (0.25 mM) for a 5 hour culture period. White bars denote absence and black bars denote presence of SMG. Data represent means of 3-5 separate yolk sacs from separate litters and are values at 5 hours. Vertical lines represent ±SEM. * Different ($p < 0.05$) from the corresponding value without SMG.

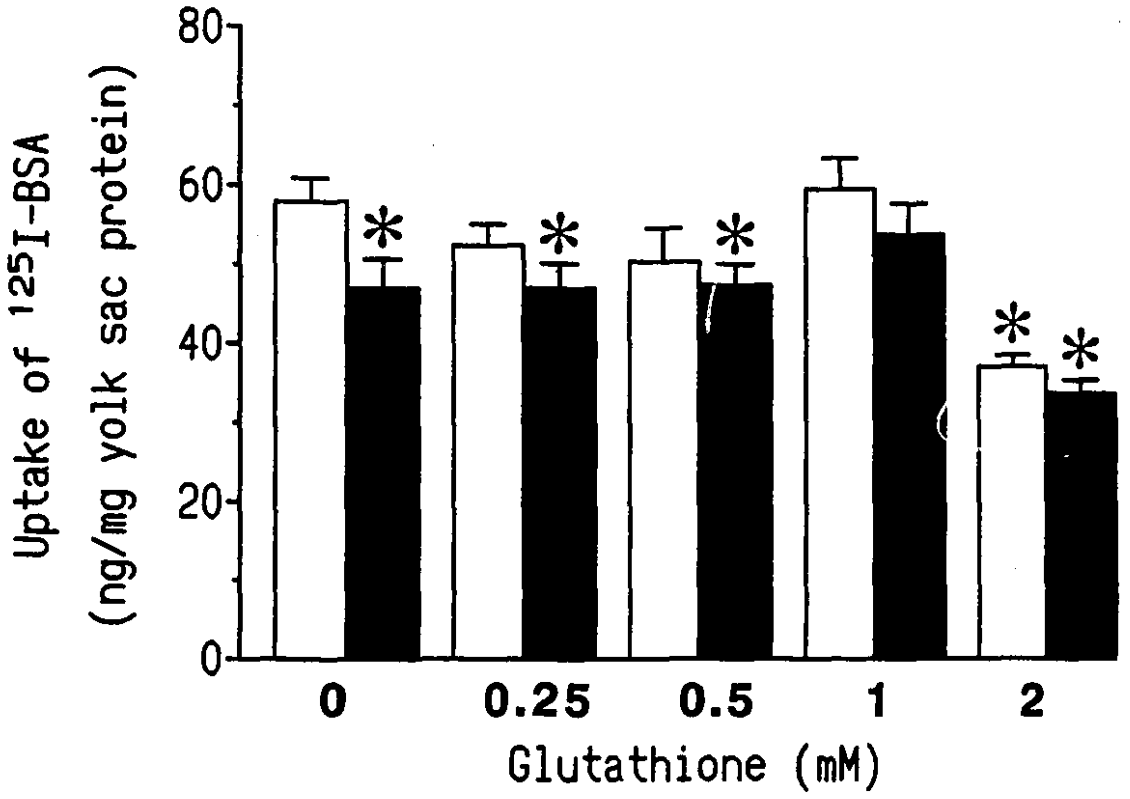

Figure 20. Effect of S-(N-methylcarbamoyl)glutathione (SMG) and glutathione (GSH) on the uptake of ^{125}I-BSA (bovine serum albumin) by isolated day 15 mouse yolk sacs. Yolk sacs were cultured individually in 10 ml medium 199. GSH was added 30 minutes before and ^{125}I-BSA (0.1 µCi/ml) 30 minutes after SMG (0.25 mM) for a 5 hour culture period. White bars denote absence and black bars denote presence of SMG. Data represent means of 3-5 separate yolk sacs from separate litters and are values at 5 hours. Vertical lines represent ±SEM. * Different ($p < 0.05$) from the corresponding value without SMG.

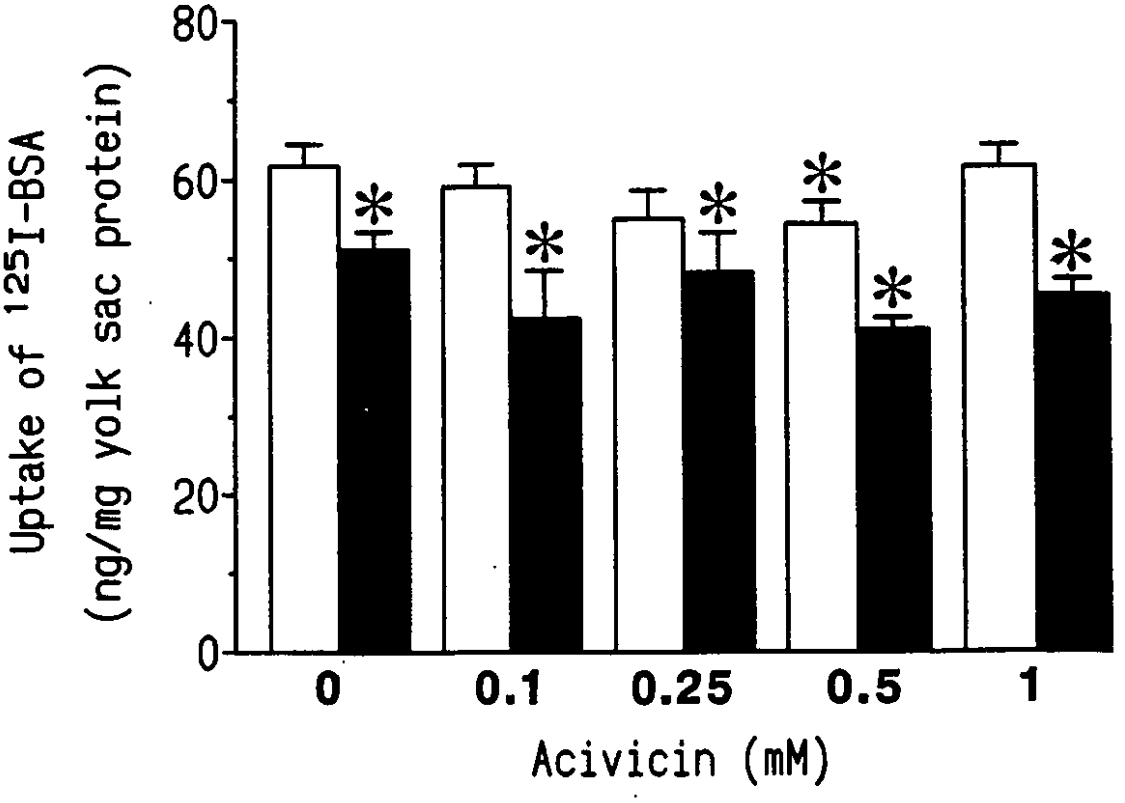

Figure 21. Effect of S-(N-methylcarbamoyl)glutathione (SMG) and acivicin on the uptake of ^{125}I-BSA (bovine serum albumin) by isolated day 15 mouse yolk sacs. Yolk sacs were cultured individually in 10 ml medium 199. Acivicin was added 30 minutes before and ^{125}I-BSA (0.1 μCi/ml) 30 minutes after SMG (0.25 mM) for a 5 hour culture period. White bars denote absence and black bars denote presence of SMG. Data represent means of 3-5 separate yolk sacs from separate litters and are values at 5 hours. Vertical lines represent ±SEM. * Different ($p < 0.05$) from the corresponding value without SMG.

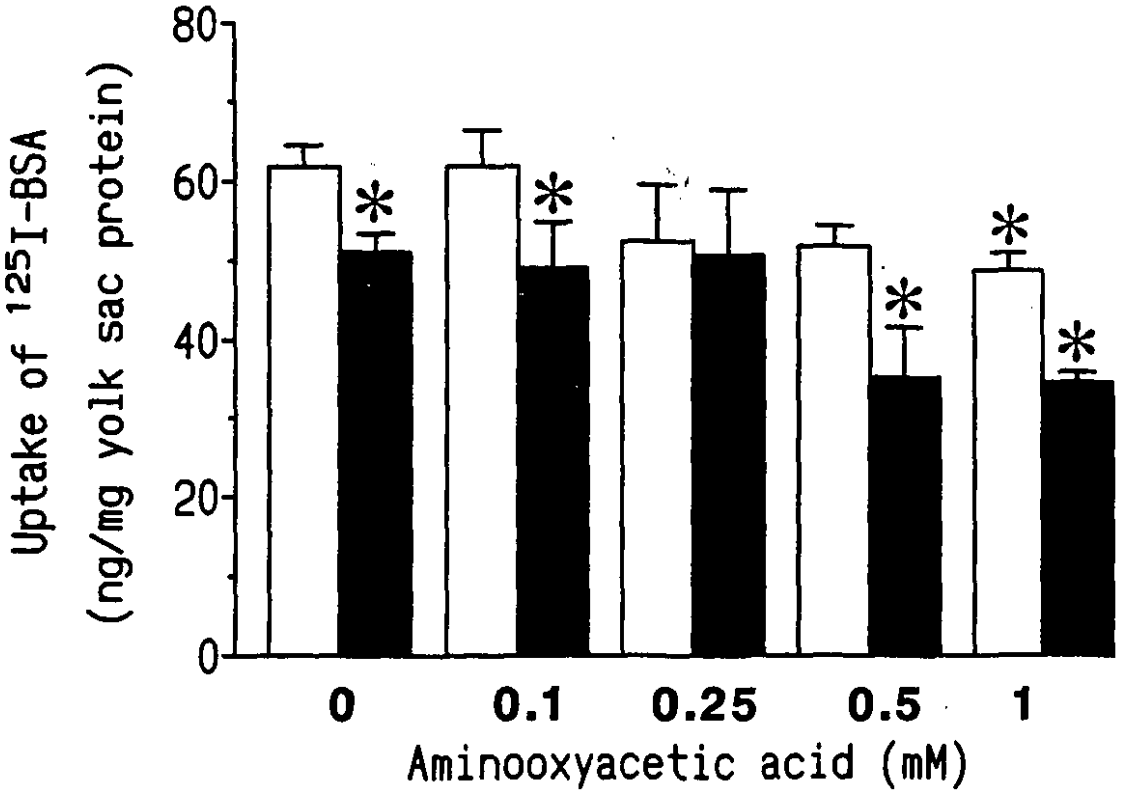

Figure 22. Effect of S-(N-methylcarbamoyl)glutathione (SMG) and aminooxyacetic acid (AOAA) on the uptake of ^{125}I-BSA (bovine serum albumin) by isolated day 15 mouse yolk sacs. Yolk sacs were cultured individually in 10 ml medium 199. AOAA was added 30 minutes before and ^{125}I-BSA (0.1 μCi/ml) 30 minutes after SMG (0.25 mM) for a 5 hour culture period. White bars denote absence and black bars denote presence of SMG. Data represent means of 3-5 separate yolk sacs from separate litters and are values at 5 hours. Vertical lines represent ±SEM. * Different ($p < 0.05$) from the corresponding value without SMG.

3.2.6 Utilization of endogenous precursors

3.2.6.1 Effects of TMA

When day 8 embryos were cultured in serum in which proteins had been labelled with ^{3}H-leucine or in serum to which ^{3}H-leucine had been added, TCA-insoluble radioactivity accumulated in the embryo and yolk sac. TMA treatment increased the amount of ^{3}H-leucine taken up and incorporated into protein in embryos and yolk sacs cultured in serum containing free ^{3}H-leucine, in comparison to untreated controls (Tables 14 and 15). TMA reduced the uptake of total radioactivity to 47% and 77% of control embryos and yolk sacs, respectively, when embryos were cultured in serum containing ^{3}H-leucine-labelled protein (Tables 16 and 17). This difference was significant only for the embryo. The amount of radioactivity incorporated into protein was also reduced by TMA (Tables 16 and 17), but again was only significant in the case of the embryo. Whether ^{3}H-leucine was supplied in the culture media as free ^{3}H-leucine or ^{3}H-leucine-labelled proteins, the percent of total radioactivity taken up that was incorporated into protein was unaffected by TMA.

The addition of zinc to TMA-treated embryos cultured in serum containing ^{3}H-leucine-labelled proteins increased both the amount of radioactivity taken up and incorporated into protein in embryos (Table 16) but not in yolk sacs (Table 17). Despite this effect, total radioactivity taken up and the amount incorporated into protein in cultures treated with TMA and zinc was still less than untreated controls, though this difference was not significant. Embryonic and yolk sac protein was increased by zinc in TMA-treated embryos (Tables 16 and 17); the increase was significant in embryos. However, protein content of both tissues was still less than that in untreated controls; in the case of embryos this difference was significant.

Table 14. Effect of trimethylamine (TMA) on the incorporation of ^{3}H-leucine into proteins of mouse embryos cultured in serum containing ^{3}H-leucine

Variables	Control	TMA (0.75mM)
Leucine uptake (fmol)	904±62	1174±150
(Leucine/embryo)/(leucine/µl medium)	26.9±3.9	9.7±2.1*
Leucine incorporated (fmol/mg protein)	291±15	334±28
Total protein/embryo (µg)	41.7±5.2	11.1±1.6*

Day 8 embryos were cultured in 90% serum:10% saline. ^{3}H-Leucine (0.25 µCi/ml) was added 8 hours after explantation for a 16 hour labelling period. TMA was added 30 minutes after the beginning of culture and was present for the entire culture period. Uptake refers to total ^{3}H-leucine/mg embryonic protein. Data represent means of 5 separate embryos from 5 separate litters. * Different ($p < 0.05$) from the corresponding control value.

Table 15 . Effect of trimethylamine (TMA) on the incorporation of ^{3}H-leucine into proteins of mouse yolk sacs cultured in serum containing ^{3}H-leucine

Variables	Control	TMA
Leucine uptake (fmol)	649±100	672±20
(Leucine/yolk sac)/ (leucine/µl medium)	17.25±1.8	14.46±2.7
Leucine incorporated (fmol/mg embryonic protein)	210±23	227±11
Total protein/yolk sac (µg)	44.8±2.3	31.0±6.2*

Day 8 embryos were cultured in 90% serum:10% saline. ^{3}H-Leucine (0.25 µCi/ml) was added 8 hours after explantation for a 16 hour labelling period. TMA (0.75 mM) was added 30 minutes after the start of culture and was present for the entire culture period. Uptake refers to total ^{3}H-leucine/mg yolk sac protein. Data represent means of 5 separate yolk sacs from separate litters. * Different ($p < 0.05$) from the corresponding control value.

Table 16. Effects of trimethylamine (TMA) and zinc on the incorporation of ^{3}H-leucine into proteins of mouse embryos cultured in serum containing ^{3}H-leucine-labelled proteins

Variables	Control	TMA	Zinc	TMA+Zinc
Leucine uptake (fmol)	61.2±9.1	28.7±1.9*	76.9±11.8	31.4±3.3
(Leucine/embryo)/ (leucine/µl medium)	1.04±0.17	0.12±0.02*	1.51±0.46	0.33±0.07
Leucine incorporated (fmol/mg embryonic protein)	20.3±3.5	8.9±1.8*	29.5±5.9	13.5±1.3
Total protein/embryo (µg)	64.5±3.8	18.8±1.7*	55.6±6.8	26.1±3.6*#

Day 8 embryos were cultured in unlabelled serum for 8 hours then transferred to the radiolabelled serum. Zinc was added 30 minutes and TMA 60 minutes after the start of culture and both were present for the entire culture period. Uptake refers to total ^{3}H-leucine/mg embryonic protein. Means represent 5-9 separate embryos for each treatment from 5-9 separate litters. TMA, 0.75 mM; zinc, 76 µM. * Different ($p < 0.05$) from the corresponding control value and the value for zinc. # Different ($p < 0.05$) from the corresponding value for TMA alone.

Table 17. Effects of trimethylamine (TMA) and zinc on the incorporation of ^{3}H-leucine into proteins of mouse yolk sacs cultured in serum containing ^{3}H-leucine-labelled proteins

Variables	Control	TMA	Zinc	TMA+Zinc
Leucine uptake (fmol)	79±8	61±10	75±10	53±7
(Leucine/yolk sac)/ (leucine/µl medium)	1.1±0.07	0.26±0.03*	1.28±0.3	0.83±0.3
Leucine incorporated (fmol/mg yolk sac protein)	24.8±3.4	19.3±3.2	24.0±2.6	17.9±2.0
Total protein/yolk sac (µg)	51.2±4.6	29.0±5*	45.5±5.7	40.0±7.0

Day 8 embryos were cultured in unlabelled serum for 8 hours then transferred to the radiolabelled serum. Zinc was added 30 minutes and TMA 60 minutes after the beginning of culture and both were present for the entire culture period. Uptake refers to total ^{3}H-leucine/mg yolk sac protein. Means represent 5-9 separate yolk sacs for each treatment from separate litters. TMA, 0.75 mM; zinc, 76 µM. * Different ($p < 0.05$) from the corresponding control value and the value for zinc.

3.2.6.2 Effects of SMG

When day 8 embryos were cultured in serum containing free ^{3}H-leucine, SMG caused a reduction in the amount of radioactivity taken up and incorporated into proteins of both embryos (Table 18) and yolk sacs (Table 19). The amount incorporated in embryos but not the amount taken up was significantly reduced. Total protein per yolk sac but not per embryo was also significantly reduced.

When SMG-treated embryos were cultured in serum containing ^{3}H-leucine-labelled proteins, the amount of ^{3}H-leucine taken up and incorporated into proteins was significantly reduced (Tables 20 and 21). The amount of radioactivity taken up was 47% and 49%, respectively, of uptake in control embryos and yolk sacs. The incorporation rate in SMG-treated embryos was 51% and in yolk sacs 65% of corresponding control values. The protein content of both embryos and yolk sacs was significantly less than that in controls.

3.2.7 Limb bud cultures

3.2.7.1 Effects of TMA

Incubation of isolated day 12 limb buds in serum to which ^{3}H-leucine had been added resulted in the appearance of TCA-insoluble radioactivity in the limb buds. TMA did not alter the total amount of radioactivity taken up, the amount incorporated into protein or the protein content per limb bud (Table 22).

3.2.7.2 Effects of SMG

SMG did not affect the uptake of ^{3}H-leucine by day 12 limb buds (Table 23). The amount of ^{3}H-leucine incorporated into protein and the total protein content was reduced by SMG but neither reduction was significant (Table 23).

Table 18. Effect of S-(N-methylcarbamoyl)glutathione (SMG) on the incorporation of ^{3}H-leucine into protein of mouse embryos cultured in serum containing ^{3}H-leucine

Variables	Control	SMG
Leucine uptake (fmol/mg embryonic protein)	920±78	767±131
(Leucine/embryo)/(leucine/µl medium)	27.65±4.9	14.8±0.8*
Leucine incorporated (fmol/mg embryonic protein)	283±16	197±14*
Total protein/embryo (µg)	45.5±5.1	33.3±5.4

Day 8 embryos were cultured in 90% serum:10% saline. ^{3}H-Leucine (0.25 µCi/ml) was added 8 hours after explantation for a 16 hour labelling period. SMG (0.25 mM) was added 30 minutes after the beginning of culture and was present for the entire culture period. Data represent means of 5 separate embryos from 5 separate litters. * Different ($p < 0.05$) from the corresponding control value.

Table 19. Effect of S-(N-methylcarbamoyl)glutathione (SMG) on the incorporation of ^{3}H-leucine into protein of mouse yolk sacs cultured in serum containing ^{3}H-leucine

Variables	Control	SMG (.25mM)
Leucine uptake (fmol/mg yolk sac protein)	647±115	819±70
(Leucine/yolk sac)/(leucine/µl medium)	15.9±1.4	12.4±1.1*
Leucine incorporated (fmol/mg yolk sac protein)	202±29	245±12
Total protein/yolk sac (µg)	42.9±1.8	24.2±3.1*

Day 8 embryos were cultured in 90% serum:10% saline. ^{3}H-Leucine (0.25 µCi/ml) was added 8 hours after explantation for a 16 hour labelling period. SMG was added 30 minutes after the beginning of culture and was present for the entire culture period. Data are means of 5 separate yolk sacs from separate litters. * Different ($p < 0.05$) from the corresponding control value.

Table 20. Effect of S-(N-methylcarbamoyl)glutathione (SMG) on the incorporation of ^{3}H-leucine into proteins of mouse embryos cultured in serum containing ^{3}H-leucine-labelled proteins

Variables	Control	SMG
Leucine uptake (fmol/mg embryonic protein)	55±9	26±2*
(Leucine/embryo)/(leucine/μl medium)	0.94±0.17	0.23±0.03*
Leucine incorporated (fmol/mg embryonic protein)	18.5±3.5	9.4±1.2*
Total protein/embryo (μg)	58.8±3.8	30.2±5.2*

Day 8 embryos were cultured in unlabelled serum for 8 hours then transferred to the radiolabelled serum. SMG (0.25 mM) was added 30 minutes after the start of culture and was present for the entire culture period. Data represent means of 6-7 separate embryos from 6-7 separate litters. * Different ($p < 0.05$) from the corresponding control value.

Table 21. Effect of S-(N-methylcarbamoyl)glutathione (SMG) on the incorporation of ^{3}H-leucine into proteins of mouse yolk sacs cultured in serum containing ^{3}H-leucine-labelled proteins

Variables	Control	SMG
Leucine uptake (fmol/mg yolk sac protein)	82±10	40±7*
(Leucine/yolk sac)/(leucine/µl medium)	1.06±0.07	0.25±0.03*
Leucine incorporated (fmol/mg yolk sac protein)	24.3±3.9	15.7±0.5*
Total protein/yolk sac (µg)	48.8±3.1	18.5±2.0*

Day 8 embryos were cultured in unlabelled serum for 8 hours then transferred to the radiolabelled serum. SMG (0.25 mM) was added 30 minutes after the beginning of culture and was present for the entire culture period. Data are means of 6-7 separate yolk sacs from separate litters. * Different ($p < 0.05$) from the corresponding control value.

Table 22. Effect of trimethylamine (TMA) on the incorporation of ^{3}H-leucine into proteins of day 12 mouse limb buds

Variables	Control	TMA (0.75 mM)
Leucine uptake (fmol/mg limb bud protein)	6,005±256	6,296±185
(Leucine/limb bud)/(leucine/µl medium)	27.5±1.9	29.2±1.9
Leucine incorporated (fmol/mg limb bud protein)	3,699±168	3,639±145
Total protein/limb bud (µg)	32.1±2.8	33.2±3.1

Pairs of limb buds were cultured in 75% Bigger's medium:25% serum. ^{3}H-Leucine (1 µCi/ml) was added 30 hours after the beginning of culture for a 16 hour labelling period. TMA was added 30 minutes after the beginning of culture and was present for the entire culture period. Data are means of 7-8 separate pairs of limb buds from 7-8 separate litters.

Table 23. Effect of S-(N-methylcarbamoyl)glutathione (SMG) on the incorporation of ^{3}H-leucine into proteins of day 15 mouse limb buds

Variables	Control	SMG
Leucine uptake (fmol/mg limb bud protein)	6,111±275	5,939±456
(Leucine/limb bud)/(leucine/µl medium)	28.7±1.7	24.8±0.7*
Leucine incorporated (fmol/mg limb bud protein)	3,718±197	3,410±214
Total protein/limb bud (µg)	32.8±3.2	26.5±2.6

Pairs of limb buds were cultured in 75% Bigger's medium:25% serum. ^{3}H-Leucine (1 µCi/ml) was added 30 hours after the beginning of culture for a 16 hour labelling period. SMG (0.25 mM) was added 30 minutes after the beginning of culture and was present for the entire culture period. Data are means of 4-5 separate pairs of limb buds from 4-5 separate litters.
* Different ($p < 0.05$) from the corresponding control value.

CHAPTER FOUR
DISCUSSION

4.1 Scope

The results obtained from experiments conducted *in vivo* form the first part of the discussion. The toxicity of trimethylamine (TMA) in comparison to other methylamines is discussed, along with possible reasons why TMA is selectively toxic to male offspring of TMA-treated mice. The lack of toxicity of S-(N-methylcarbamoyl)glutathione (SMG) *in vivo* is also discussed.

The second part contains a discussion on the effects of TMA and SMG on embryo cultures, possible reasons for the lack of antagonistic action of antioxidants, thiol compounds and growth factors against TMA-induced embryotoxicity and the embryotoxicity of SMG is compared to other isocyanate/isothiocyanate conjugates and to SMG toxicity in cell cultures. Although the headings *in vivo* and *in vitro* separate the first and second parts of the discussion, these are mainly to orient the reader. The *in vitro* experiments are an attempt to isolate some aspects of the *in vivo* system while maintaining others, and a discussion and the relevance of *in vitro* data cannot be too rigorously separated from data collected *in vivo*.

The effects of TMA and SMG on uptake mechanisms of the yolk sac are discussed next. The possible role of the lysosomotropic action of TMA in the inhibition of uptake is mentioned. The approaches to antagonize SMG-induced inhibition of uptake are discussed. The utilization of endogenous precursors by embryos in the presence of TMA and SMG is also discussed.

Finally, conclusions and clinical relevance are presented. The importance of SMG in relation to the Bhopal accident is discussed. Disease states are mentioned in which serum

methylamines are increased and the relevance of the selective toxicity of TMA to male offspring is discussed in light of the pathology of clinical disease.

4.2 Detailed discussion

In vivo

The main findings *in vivo* were that, of the three methylamines tested, only TMA produced foetal toxicity, in the form of reduced foetal body weight. There was no evidence of teratogenesis. TMA administration to dams during gestation resulted in a selective inhibition of the postnatal growth of male offspring. SMG did not produce any foetal toxicity *in vivo*.

The tremors, ataxia and tonic convulsions observed in mice after administration of methylamines at greater than 1 mM is probably due to the amine moiety. Dogs eating large amounts of shark meat, known to contain high concentrations of TMA, also demonstrate hypersalivation, tremors, incoordination and convulsions (Anthoni *et al.*, 1991). Lethargy, stupor, convulsions and disorientation have been observed in a patient with trimethylaminuria (Danks *et al.*, 1976) and it has been suggested that TMA may contribute to the pathogenesis of hepatic encephalopathy, in which ammonia is a suspected causative agent (Marks *et al.*, 1978).

A single injection of the methylamines or dimethylurea into pregnant mice (Table 1) had no effect on pregnancy development. This suggests that the reproductive toxicity observed in Bhopal survivors was not due to the conversion of MIC to these metabolites after an acute exposure to MIC vapour.

The death of virgin females administered TMA at 5 mmol/kg/day was preceded by weight loss, in contrast to deaths in pregnant mice, which were not preceded by any effect

on body weight. Since the initial body weight in the two groups was comparable and weight gain in the pregnant mice in the first few days was not excessive (all virgin mice had died by the ninth day), the different sequelae are unlikely to be due to reduced dose per unit body weight in pregnant animals. Pregnancy-induced changes in hormonal status, drug metabolism and volumes of distribution can affect the toxicity of xenobiotics. Another possible explanation for the reduced toxicity of TMA in pregnant mice in comparison to virgin mice is an increase in binding of TMA to pregnancy-associated serum proteins. The development of pregnancy in rats and mice is accompanied by a 100-fold increase in the serum levels of α_2-macroglobulin, the rodent equivalent of human pregnancy zone protein (Ganrot, 1973; Saito and Sinohara, 1985). Pregnancy zone protein (PZP), α_2-macroglobulin and the structural analogous complement components C3 and C4 have in common binding and inactivation by methylamine (Sim *et al.*, 1981; Gorski and Howard, 1980; Sand *et al.*, 1985). Although only primary amines inactivate C3 or C4, secondary and tertiary amines do bind to both C4 and PZP. If mouse α_2-macroglobulin has similar binding properties to human α_2-macroglobulin then the binding of TMA to this and possibly other serum proteins may be sufficient to reduce the intracellular level of TMA below pathological concentrations.

The specific effect of TMA to induce foetal growth retardation *in utero* is likely to be a consequence of chemical and pharmacokinetic characteristics that distinguish TMA from MMA and DMA. The pKa values for MMA, DMA and TMA are 10.7, 10.8 and 9.7, respectively. This means that at a pH of 7.4, the ratio of nonionized to ionized forms of these three molecules would be 1,778, 1,906 and 240, respectively. Thus, at pH 7.4 there is an 8-fold greater amount of nonionized TMA in comparison to MMA or DMA. Only the nonionized form crosses cell membranes freely, so on average there would be an 8-fold greater amount of TMA than MMA or DMA entering cells. A higher ratio of intracellular to

extracellular concentration of TMA than of DMA has been recorded (Ihle *et al.*, 1984). Furthermore, once inside a cell, compounds with a high pKa may take hours to days to equilibrate between the cytoplasm and the lysosome while those with a lower pKa value will equilibrate much more rapidly (Dean *et al.*, 1984). TMA can therefore be expected to enter the lysosome and other acidic organelles more quickly than DMA or MMA. The consequences of this are discussed below.

A difference in the pH between the maternal and foetal compartments may also contribute to the selective foetal toxicity of TMA. The pH of the foetal compartment during the early part of organogenesis (day 8-11) in mice is 0.1-0.4 pH units above the maternal serum pH (Nau and Scott, 1986). Thus more unionized trimethylamine would be present in the foetus than in the mother.

Another factor that could contribute to the selective foetal effect of TMA is that at high doses, the metabolic pathway for TMA may be overwhelmed. The conversion of TMA to trimethylamine-N-oxide (TMAO) can be considered a detoxication step; TMAO is approximately 10-fold less toxic than TMA. The oxidation of TMA is rate-limiting at high doses in humans (Al-Waiz *et al.*, 1987a). Assuming that this is also true in mice, the doses administered to pregnant animals very likely overwhelmed the capacity of the dams to oxidize TMA. This would extend the half-life of TMA in the maternal serum and so increase the length of time the foetus is exposed to high TMA concentrations. Similarly high doses of MMA and DMA would not be of the same consequence, because of the chemical differences pointed out above and presumably because, being endogenous compounds, their metabolic and excretory pathways (DMA is excreted unchanged) are more efficient at handling high doses.

Several pieces of evidence suggest an increased susceptibility of male offspring of

TMA-treated mice to the toxicity of TMA in comparison to females. TMA given throughout gestation (2.5 mmol/kg/day) or only during organogenesis (5 mmol/kg/day) caused a significant decrease in male but not female progeny body weights. Furthermore, only in male offspring was there a significant depression in brain weight, protein and DNA content. The highest dose of TMA (7.5 mmol/kg/day) did reduce body weight in both sexes, but the decrease was greater in males (Figure 2). The reduced brain and kidney weights most likely represent reduced body growth and not a specific effect on the ontogeny of these organs, since the weights as a proportion of body weight were not changed. However, it is interesting to note that at the dose of 5 mmol/kg/day, the kidney was the only organ besides the seminal vesicle that was reduced in weight. One possible explanation is that TMA, via its lysosomotropic action (Seglen and Gordon, 1980), exerts a direct effect on the kidney, which is known to possess active primary and secondary lysosomes during its maturation (Schaeverbeke and Cheignon, 1980).

The failure of 8 and 14 week-old offspring of TMA-treated mice to attain control body weights is suggestive of a permanent interference in the potential for growth. The data on cellular protein and DNA content supports the view that this could be the result of a decrease in cell number. For example, between 3 and 8 weeks of age, brain DNA and protein of female offspring of mice treated with TMA at 7.5 mmol/kg/day during organogenesis increased to levels equal to or greater than those observed in controls (Table 6). In contrast, in male offspring, the levels of brain DNA and protein failed to attain those of controls. DNA or protein per unit tissue weight was not reduced and the protein/DNA ratios were similar to controls, suggesting that exposure to TMA resulted in a reduction in cell number rather than in cell size.

The male-specific inhibition of postnatal growth may be due to the greater rate of

growth in males than in females. It has been reported previously that TMA inhibits the growth of male but not female rats (Anonymous, 1980). The age of the rats was not specified but they are presumed to be young adults, in order that changes in body weight were measurable. No suggestion was made as to the underlying cause. The greater rate of growth in males is believed to be due to testosterone which, in part by regulating somatostatin and growth hormone releasing hormone gene expression, results in pulsatile secretion of growth hormone from the pituitary (Werner *et al.*, 1988; Chowen-Breed *et al.*, 1989; Zeitler *et al.*, 1990; Painson and Tannenbaum, 1991). This episodic release of growth hormone distinguishes and characterizes male growth (Jansson *et al.*, 1985).

The data on body and organ weights and serum testosterone levels in 8 and 14 week-old male mice support the hypothesis of reduced testosterone in male progeny of TMA-treated mice. However, serum testosterone was only collected from progeny of mice exposed to the highest dose of TMA. An assessment of serum testosterone at different dose levels of TMA would have been desirable.

One possible way in which TMA could be reducing testosterone levels would be by interfering in its synthesis. Monensin and chloroquine, two lysosomotropic drugs which have several biochemical endpoints in common with TMA, including inhibition of receptor recycling and of lysosomal proteolysis, have been shown to inhibit steroidogenesis, possibly by inhibiting cholesterol transfer from the cytoplasm to the mitochondria, the rate-limiting step in steroidogenesis (Magalhaes *et al.*, 1991; Almahbobi *et al.*, 1992), or by inducing a deficiency of newly synthesized mitochondrial proteins (Cheng *et al.*, 1990). The lysosomal effects of TMA may thus reduce the synthesis of testosterone.

In addition to the influences of testosterone, growth hormone gene expression is known to be regulated by retinoic acid and thyroid hormone (Yaffe and Samuels, 1984; Bedo *et al.*,

1989). Thus an interference in either retinoic acid or thyroid hormone could conceivably reduce growth in a sexually dimorphic manner. Ammonium chloride has been shown to inhibit the secretion of thyroid hormone *in vitro* by an inhibition of thyroglobulin hydrolysis in lysosomes (Unger *et al.*, 1985; Yamashita *et al.*, 1990). The lysosomotropic action of TMA could result in an inhibition of thyroid hormone secretion and is another possible way by which TMA could be reducing male growth.

Breeding of progeny of mice treated with TMA (2.5 mmol/kg/day) throughout gestation resulted in normal rates of pregnancy, indicative of normal fertility in both males and females. Therefore, although the reduced seminal vesicle weights in the male offspring of these mice may reflect decreased serum testosterone, any decrease was not of sufficient magnitude to impair fertility.

Sexual dimorphism in the susceptibility of animals to toxic agents may occur by other known mechanisms, including sex hormone-dependent metabolism of the toxin (Smith *et al.*, 1984) and the presence of sex-specific compounds, such as α_{2U}-globulin (Vandoren *et al.*, 1983). Lithium administration and heat stress to pregnant animals results in selective toxicity to male offspring and it has been suggested that this effect of lithium may involve a disturbance of the endocrine system (Mroczka *et al.*, 1983; Shiota and Kayamura, 1989). There is therefore a possibility that TMA is selectively toxic to male offspring in a manner unrelated to testosterone.

The administration of SMG to pregnant mice treated with buthionine sulfoxime (BSO) was expected to result in some deleterious effect on pregnancy outcome. It is not known why no adverse effects were observed. An increase in glutathione (GSH) concentration by exogenous supply of GSH or precursors, or depletion of GSH by BSO or diethyl maleate has been amply demonstrated to protect or attenuate the toxicity of chemicals that are

metabolized via GSH conjugation or the mercapturic acid pathway. The teratogenicity of cyclophosphamide and the toxicity of 1,2-dichloropropane and N-methylformamide are increased by prior depletion of GSH (Hales, 1981; Imberti *et al.*, 1990; Tulip and Timbrell, 1988). The toxicity of these agents is believed to be due, in part, to a depletion of cellular GSH. The toxicity of α-naphthylisothiocyanate, a chemical that forms a reversible conjugate with GSH, is mediated through GSH conjugation, and protection against the cellular toxicity of α-naphthylisothiocyanate can be achieved by prior depletion of GSH with BSO (Dahm and Roth, 1991). Indeed, indices of toxicity became evident as GSH levels returned to normal (Dahm and Roth, 1991).

Glutathione has been shown to antagonize the toxicity of SMG in cell (Han *et al.*, 1990) and embryo (Guest *et al.*, 1992) cultures. By inference, administration of a reversible glutathione conjugate after pretreatment with BSO would be expected to exacerbate any toxicity, given release of the electrophile from GSH. That no effect was observed may be due to low release of MIC from SMG. Although release is favoured in low GSH environments, pH is also an important factor; dissociation of GSH conjugates is more likely at alkaline pH (Bruggeman *et al.*, 1986). It is also possible that the dose was not high enough to elicit toxicity or that other serum proteins that increase during pregnancy acted as nucleophilic centres to bind any MIC released from SMG, preventing binding and toxicity at other sites.

In vitro

The decreased growth of embryos *in vitro* after removal from dams acutely exposed to MIC vapour suggests that either, due to maternal toxicity (including a non-specific stress response), MIC acted very quickly to inhibit the potential for foetal growth or that MIC was delivered to the foetal space in some form to act directly on the foetus. One cannot

determine from the results presented here which occurred, but evidence from other investigators is consistent with the second possibility. Inhalation of ^{14}C-methyl isocyanate by pregnant mice resulted in rapid labelling of many tissues, including the foetus (Ferguson *et al.*, 1986) and inhalation of ^{14}C-MIC by rats led to extensive covalent binding to various tissues within 30 minutes (Bhattacharya *et al.*, 1988). The exact nature of the radioactivity was not determined but the results suggest passage and transport of MIC by the systemic circulation.

Pregnant mice exposed to MIC vapour for 3 hours on day 8 of gestation exhibited reduced body weight gain and increases in foetal loss (Varma, 1987). At low concentrations of MIC (< 6 ppm), foetal loss was comparable to controls but foetal weight was reduced. At higher concentrations of MIC (6-15 ppm), 80% of dams lost the entire litter (Varma, 1987). The reduced weight gain and loss of whole litters in MIC-exposed dams are indicative of maternal toxicity but the reduced embryonic growth *in vitro* after a 2 hour exposure *in utero* would suggest direct action of MIC on the foetus.

The fact that injection of MIC into the sealed atmosphere of culture bottles resulted in embryonic death in mice and reduced growth of rat embryos (Varma *et al.*, 1990) confirms that MIC is directly foetotoxic. Although it had been shown that lung toxicity was not necessary in order to produce foetal toxicity (Varma, 1987), it was not then known if foetal toxicity was simply the result of maternal toxicity. This result demonstrates that the maternal and foetal toxicity of MIC can be dissociated. Since the concentration of MIC in the bottles rapidly (within 10 minutes) diminished, MIC either exerted its toxicity very quickly or was transformed/metabolised in the culture media to another form capable of retaining its toxicity. The embryo culture media is 90% serum, which contains high concentrations of glutathione, so the formation of S-(N-methylcarbamoyl)glutathione is quite possible.

In contrast to *in vivo*, all three methylamines were embryotoxic *in vitro*, producing concentration-dependent decreases in growth and development. SMG also produced concentration-dependent decreases in growth and both TMA and SMG were teratogenic.

Although the precise nature of the disparity between the *in vivo* and the *in vitro* data is not known, it may be due to a lack of partitioning between a maternal and a foetal unit, which may have acted to concentrate the amines in the foetus. Even so, TMA was more toxic than DMA or MMA, perhaps due to a greater intracellular concentration, as is predicted from the pKa values.

Aqueous solutions of the three methylamines are weakly basic but sufficient buffering capacity existed in the embryo culture media so that concentrations up to 2 mM did not alter the pH. The possibility that pH changes were the cause of the observed growth reduction is therefore unlikely. The addition of 1-2 mM glycerol and glucose to the culture media did not produce adverse effects on embryonic growth, suggesting that changes in osmolarity were also not involved in the growth inhibition induced by the methylamines.

The appearance of yolk sacs in which reduced transparency had been induced by DMA and TMA has been reported in isolated hepatocytes cultured in the presence of TMA (Seglen and Gordon, 1980). These authors attributed this effect to the build-up of undigested protein in the cell lysosomes but vacuolation and the osmotic uptake of water into lysosomes of methylamine-treated cells has also been cited as a cause of reduced transparency (Ohkuma and Poole, 1981). The concentration used in those cell culture experiments was 10 mM, whereas the concentration in the embryo cultures eliciting this translucency in yolk sacs was 1-2 mM. If the translucency was due to an effect on lysosomes or vacuoles, the difference in concen-tration can be explained by the proliferative nature of the embryo. Rapidly dividing tissues have been noted to be much more susceptible to the toxicity of methylamines than

quiescent cells (Cain and Murphy, 1986) and so symptoms that were noted in cell cultures would be expected to occur in developing embryos at lower concentrations.

A dose of 0.75 mM TMA was chosen from the initial concentration-response studies because at this concentration, TMA elicited sub-maximal (50-75%) toxicity, about 30% mortality and permitted embryos to grow throughout the 42 hours of culture. The dorsal-rostral split in the heads of embryos treated with ≥ 0.75 mM TMA would appear to be arrest of a normal process rather than the specific induction of a dysmorphogenic event. At the time of explantation, equivalent to day 8 of gestation, the neural folds are divided along the midline. During the next 24 hours these folds fuse together, forming the fore-, mid- and hindbrain. The interruption of neural fold fusion induced by TMA was consistently produced in embryos that had 4-6 somites at the beginning of culture but was much less reproducible if embryos of 4-8 somites were used. A similar maturation-stage dependence of neural tube defects has been noticed with other chemical agents. Valproic acid and 7-hydroxy-2-acetylaminofluorene both cause open neural tubes in rat embryos; embryos with 6-10 somites were most susceptible (Harris *et al.*, 1988). This stage is later than that observed with TMA in mouse embryos and may be due to species variation.

Widely diverse chemical agents and physical stresses can induce neural tube defects, including heat (Cockcroft and New, 1975), fasting (Runner and Miller, 1956), hyperglycaemia (Cockcroft and Coppola, 1977), valproic acid, 7-hydroxy-2-acetylaminofluorene and cytochalasin D (Harris *et al.*, 1988) and inositol deficiency (Cockcroft, 1991). This would suggest that the induction of neural tube defects is a consequence of the highly proliferative nature of the tissue rather than specific actions of the various agents. The fact that later stage embryos, in which neural tube formation and closure is almost complete, are much less susceptible to chemical agents or vitamin deficiencies than earlier stage embryos

supports this view (Harris *et al.*, 1988; Cockcroft, 1988). A delay in cell division, due to interruption of energy or nutrient supply or cell death, could be expected to interfere in normal cell migration that determines the morphology of the brain (Copp *et al.*, 1990). There are recent reviews on both animal and human studies of neural tube defects (Campbell *et al.*, 1986; Copp *et al.*, 1990). A recent epidemiological study concluded that folic acid supplementation during pregnancy had significant protective effect against neural tube defects in infants of high risk mothers (MRC Vitamin Study Research Group, 1991). The first links between folic acid deficiency and neural tube defects were noted over 40 years ago (Giroud and Boisselot, 1951) but the mechanism underlying the protective effect remains unclear.

Daily administration of TMA into mice for the entire duration of pregnancy caused a significant decrease in foetal body weight but no abnormalities were detected (Guest and Varma, 1991). It would thus seem that either the abnormal neural tube closure observed *in vitro* did not occur *in vivo* or the affected embryos died *in utero*; preliminary data support the former. Offspring of TMA-treated mice were followed for up to 14 weeks and normal overt behaviour would suggest the absence of any neural tube defect. Furthermore, although a qualitative analysis was not done, there was no difference in the gross appearance of the brain at autopsy between the untreated and TMA-treated mice.

The inhibitory effects of 0.75 mM TMA on morphologic development of embryos and on embryonic DNA, RNA and protein content reveal that the effects of TMA on growth are time-dependent and exerted after a relatively short period of time. For example, within 2 hours of the addition of TMA, the incorporation of ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-leucine into, respectively, DNA, RNA and protein, was less than that in controls, and this difference attained statistical significance at 4-8 hours (Figures 5-7). Likewise, a significant effect on

morphological development was observed by 4 to 8 hours after the addition of TMA.

The observation that the incorporation of all the three precursors into their respective macromolecules was inhibited at the same time and to almost the same extent suggests that the effects of TMA on protein synthesis are not a consequence of its effects on DNA and RNA synthesis; it would seem that TMA inhibits the synthesis of all three macromolecules either by different independent mechanisms or by some common mechanism, including interference with energy status, reduction in precursor (nucleotide and/or amino acid) pools and inhibition of lysosomal enzyme activity (Holbrook, 1980; Seglen *et al.*, 1979).

The data suggest that TMA slows the rate of embryonic development since TMA-treated embryos were one to two stages behind untreated controls in most categories according to the Brown and Fabro scoring system (Brown and Fabro, 1981). The findings that the inhibitory effects of TMA on morphologic development and macromolecular synthesis increase with time indicate that TMA exerts its effects at all stages of embryonic development. These inhibitory effects appear temporally related, so it would seem that a decrease in morphologic growth results from a decrease in macromolecular synthesis.

Aside from a reduction in the size, macromolecular synthesis and content and neural tube defects, the main abnormality in TMA-treated embryos was persistence of ventral convexity (Table 9). Neural tube defects were unrelated to persistence of ventral convexity; the incidence was similar in ventrally and dorsally convex embryos. The persistence of ventral convexity may reflect delayed development of the whole embryo, rather than an effect specific to flexure. TMA-treated embryos that remained ventrally convex at 42 hours did not exhibit morphology of time-matched controls; they were comparable to normal embryos that had not progressed from ventral convexity to dorsal convexity. Thus they had an appearance of arrested development.

The observed inability of cysteine, glutathione and ascorbic acid to modify the embryotoxic effects of TMA (Table 10) is consistent with the suggestion that TMA toxicity is not exerted through a depletion of glutathione or generation of metabolites and free radicals.

SMG caused a dose-dependent decrease in embryonic growth within a concentration range of 0-2 mM and dysmorphogenesis, expressed as a characteristic kinking of the spine and separation and divergence of the somites. However, unlike the methylamines, SMG-induced growth inhibition was not accompanied by significant mortality. The presence of a beating heart, even in embryos exposed to 2 mM SMG, which did not develop beyond the 4-8 somite stage i.e. no increase in yolk sac diameter or embryonic size, was an obvious sign of viability but the rest of the primitive embryo maintained integrity, even though there was no differentiation, suggesting continuous oxygenation and energy supply to the tissues throughout the 42 hours of culture. Treatment with any of the three methylamines at 2 mM resulted in 100% mortality; embryonic tissue began to disintegrate in 2 mM TMA.

From the initial concentration-response studies, 0.25 mM SMG was chosen for future experiments. This concentration reduced growth to approximately 70% of control growth. The spinal kinking observed in 38% of the mouse embryos treated with 0.25 mM SMG is similar to that reported in rat embryos following exposure to metabolites of N-hydroxy-2-acetylaminofluorene such as nitrosofluorene (Faustman-Watts *et al.*, 1986; Harris *et al.*, 1987), although these reports made no mention of somite separation or axial twisting as was observed with SMG. The spinal site affected by SMG is the point of flexure for the ventral to dorsal switch and would be expected to contain intensely active cells. Both MIC and SMG are known to carbamylate tissue proteins (Bhattacharya *et al.*, 1988; Pearson *et al.*, 1991). Carbamylation of proteins involved in this process could disrupt the alignment and symmetry of the spine and lead to increased numbers of embryos remaining ventrally convex, as was

observed.

It is apparent that DNA synthesis in the yolk sac of SMG-treated embryos occurs at a lower rate than in the embryo (Figures 10, 11). Glutathione conjugates like SMG cross cell membranes only poorly (Meister, 1988) so the yolk sac is likely to be the first target of SMG in the embryo culture system. Since a functional yolk sac is necessary for normal development, it is quite possible that SMG-induced toxicity to the yolk sac was sufficient to inhibit growth of the embryo. Embryotoxicity secondary to effects on yolk sac has been demonstrated (Brent *et al.*, 1971; Daston *et al.*, 1991), so the effects of SMG may have resulted entirely or partly from its effects on yolk sac function. However, recent data on the toxicity of SMG is consistent with free MIC, which can be released from SMG at physiological pH and temperature, crossing cell membranes (Han *et al.*, 1990). The possibility that MIC crossed the yolk sac membranes and directly inhibited embryonic development cannot therefore be ruled out.

The cysteine conjugate of MIC, which can also release MIC (Pearson *et al.*, 1990), is more likely to cross cell membranes than SMG. Low and high affinity transport systems for amino acids effectively transport cysteine conjugates into cells (Moldeus *et al.*, 1978; Schaeffer and Stevens, 1987). However, this transport system has been documented only in kidney cells and the formation of the MIC-cysteine conjugate from the glutathione conjugate has only been observed in the presence of excess (5 mM) cysteine (Pearson *et al.*, 1990). It would therefore be unlikely that significant amounts of the MIC-cysteine conjugate would be formed and/or could be responsible for most of the toxicity observed.

SMG is formed by a reversible reaction between MIC and GSH (Pearson *et al.*, 1990). GSH was not embryotoxic up to a concentration of 3 mM (Table 11). On the other hand, exposure of the culture media to MIC does result in embryotoxicity (Varma *et al.*, 1990). This

provides strong evidence that SMG exerts its toxicity by releasing MIC as was previously proposed (Pearson *et al.*, 1990; Han *et al.*, 1990). This inference is supported by the data that excess GSH was able to inhibit SMG embryotoxicity (Table 11). If this conclusion is correct, then the reversible conjugation of GSH with MIC would be an unusual example of drug metabolism serving the function of transport of a toxic molecule (Slatter and Baillie, 1991).

GSH and (-)-2-oxo-4-thiazolidine carboxylic acid (OTC) were used as thiol donors and were expected to inhibit SMG toxicity by providing molecules capable of scavenging MIC released from SMG and thereby reducing the reaction of MIC with embryonic tissues. In other words, cysteine and glutathione would act to favour association of MIC with GSH rather than dissociation of SMG to free MIC and GSH. An interaction of SMG with cysteine has been previously demonstrated (Pearson *et al.*, 1990). OTC is a cysteine pro-drug; its well-established effectiveness (Williamson *et al.*, 1982) is thought to be due to intracellular supply of GSH (Harris *et al.*, 1987). However, only GSH inhibited all the toxic effects of SMG; antagonism of SMG by OTC was limited to its effects on spinal kinking (Table 11). Perhaps the differences in the interactions of GSH, cysteine and OTC with SMG are related to the concentration of these agents which could be used in embryo cultures; higher than 0.1 mM cysteine and 4 mM OTC were toxic to the embryo. Antagonism of the toxicity of cysteine or GSH conjugates of isothiocyanates on RL-4 cells was found to require 5-20 mM cysteine and 5 mM GSH (Bruggeman *et al.*, 1986).

Concentration differences between embryonic and cell cultures also exist for the toxicity of the glutathione conjugates. Cell growth of TLX5 lymphoma cells in culture was reduced to 75% of control by 10 μM SMG and complete suppression of growth by 100 μM (Han *et al.*, 1990). Reduction of embryonic growth *in vitro* required 750 μM SMG. The cytotoxicity

of the glutathione conjugates of allyl and benzyl isothiocyanate demonstrate a similar concentration response to SMG; the LC_{50} for RL4 cells is approximately 100 μM (Bruggeman *et al.*, 1986). The greater potency of GSH conjugates in cell cultures compared to embryo cultures could be the result of higher concentrations of glutathione in the embryo culture media, which is 90% serum, compared to cell culture media, which may be 10% serum under conditions of growth stimulation.

The data on the uptake of ^{14}C-sucrose, ^{125}I-BSA, ^{3}H-leucine-labelled proteins and free ^{3}H-leucine indicate that TMA at 0.75 mM selectively inhibited receptor-mediated uptake by the yolk sac. Data on the processing of ^{125}I-BSA (bovine serum albumin) reveal that this concentration of TMA did not inhibit lysosomal degradation. Recognition of the yolk sac as a site at which agents can induce foetal toxicity came with the understanding of the nutritive role of the yolk sac. During organogenesis, the rodent yolk sac is the functional placenta, acquiring proteins, amino acids and growth factors from the culture media and delivering them in a usable form to the embryo. Although uptake is the rate-limiting step lysosomal degradation is also a critical link in the nutritive pathway, since almost all amino acids utilised by the embryo for protein synthesis are derived from proteins digested within yolk sac lysosomes (Beckman *et al.*, 1990). Consequently, an inhibition of either uptake or lysosomal degradation reduces embryonic growth and can be associated with teratogenesis (Brent *et al.*, 1971; Beck and Lowy, 1982; Daston *et al.*, 1991).

As judged by the uptake of ^{14}C-sucrose, TMA does not inhibit fluid-phase pinocytosis in day 8 embryo cultures (Figure 13) while uptake of ^{3}H-leucine-labelled proteins is clearly inhibited (Table 16). Polyacrylamide gel electrophoresis of the labelled serum revealed that albumin was the major labelled protein. By adsorption to the cell membrane, albumin and other proteins are taken up at a rate several times greater than agents that are taken up by

fluid-phase pinocytosis. Fluid-phase pinocytosis and receptor-mediated endocytosis have some features in common, such as recycling of membrane particles and fusion of vesicles with endosomes. The primary difference is the involvement of receptors. These two processes can be affected independently. Weak base lysosomotropic amines have been shown to inhibit receptor-mediated uptake without interfering in fluid-phase pinocytosis (Sando *et al.*, 1979; Wiesmann, 1974).

The fact that fluid-phase pinocytosis was not inhibited by TMA suggests that TMA probably interfered in some aspect of receptor-ligand recycling and/or processing rather than the membrane itself. There is evidence that weak base amines reduce the number of unoccupied receptors returning to the membrane from the intracellular compartment (Tietze *et al.*, 1982); receptor internalization is not affected but fusion of coated vesicles/endosomes with lysosomes and/or receptor-ligand dissociation is inhibited by methylamines because they increase lysosomal and endosomal pH (King *et al.*, 1981; Tietze *et al.*, 1980). An initial round of attachment and receptor cycling into the cell may proceed unaltered in the presence of a weak base (Kaplan and Keogh, 1981), but further receptor-mediated uptake is inhibited due to the perturbation of receptor-ligand degradation and receptor recycling. In other words, one might define the inhibition of receptor-mediated uptake induced by TMA as an inhibition of steps consequent upon or to uptake of ligands (in this case albumin) by receptors.

Bovine serum albumin is digested exclusively within lysosomes, with minimal extracellular hydrolysis (Livesay and Williams, 1979; Lloyd, 1990). Therefore, TCA-soluble counts that appear in the media are directly dependent upon the amount of ^{125}I-BSA degraded, which is directly dependent upon the amount of ^{125}I-BSA taken up. Since this is the rate-limiting step in the nutritive pathway, an expression of the ratio of TCA-soluble

counts to amount of ^{125}I-BSA taken up can be interpreted as a measure of lysosomal degradation of ^{125}I-BSA independent of uptake. A TMA concentration response plot of this ratio (Figure 16) indicates that TMA, a lysosomotropic agent, does not inhibit lysosomal degradation until the concentration exceeds 5 mM.

At the concentration used in the uptake experiments, 0.75 mM, TMA caused significant growth reduction of embryos *in vitro* (Guest and Varma, 1992). This concentration is 2-4 times less than that estimated to have been achieved *in vivo* (based on the assumption that TMA distributed into the total body water) which resulted in a 25-30% reduction in foetal growth (Guest and Varma, 1991). Thus the inhibition of uptake observed *in vitro* could explain the reduced growth *in vivo*.

It has been reported that the concentration of methylamine that inhibits growth of fibroblasts (0.7 mM) is 100-fold less than the concentration that raises the lysosomal pH one unit (Cain and Murphy, 1986). In addition, a chloroquine-induced increase in lysosomal pH of hepatocytes was transient, the pH returning to normal within 3 hours, despite the continuous presence of chloroquine (Tietz *et al.*, 1990). These data suggest that an increase in lysosomal pH induced by lysosomotropic agents may not be the main cause of the aberrant cellular physiology that occurs in their presence.

An inhibition of lysosomal proteolysis by methylamines is due to the induced increase in lysosomal pH; methylamines do not directly inhibit the enzymes (Reijngoud *et al.*, 1976). Although a strict comparison between cell and embryo cultures cannot be made, it would appear unlikely that 0.75 mM TMA, the concentration used in the uptake experiments, would cause a significant increase in the lysosomal pH. Data from the plot of lysosomal degradation of ^{125}I-BSA independent of uptake (Figure 16) support this view.

Entry of lysosomotropic agents into lysosomes produces an osmotic gradient. Water

enters the lysosome, producing vacuolation, which may physically interfere in the recycling of receptors (Schneider and Trouet, 1981). Moreover, ammonium chloride has been shown to reduce the lateral mobility of the vasopressin V_2-type receptor in the cell membrane by perturbation of the cytoskeletal microfilaments (Jans *et al.*, 1990) independent of any effects on pH. Microtubules have also been shown to play a role in endosome recycling (Goltz *et al.*, 1992). Thus, biochemical effects of TMA other than an increase in lysosomal pH could explain a reduction in receptor-mediated uptake.

The increase in uptake of ^{125}I-BSA after a preincubation with 5 mM TMA is probably a reflection of inhibition of lysosomal proteolysis. At this concentration, lysosomal degradation of ^{125}I-BSA is inhibited to a greater extent than receptor-mediated uptake, so that acid-insoluble radioactivity accumulates in the yolk sac. When the TMA is removed, lysosomal proteolysis returns to normal and degrades the accumulated ^{125}I-BSA at the same rate as untreated yolk sacs, as evidenced by the parallel plots of degradation versus time (Figure 15). Such a phenomenon, of increased uptake of ^{125}I-BSA at low concentrations of a lysosomotropic agent followed by reduced uptake at high concentrations, has been noted in rat yolk sacs exposed to ammonium chloride (Livesay *et al.*, 1980).

An attempt was made to antagonize the growth inhibition of TMA by the addition to the cultures of various growth factors known to be essential for normal development. The choice of a diverse group of the growth factors was deliberate, in order to determine if TMA inhibited a common pathway. An inhibition of receptor recycling or ligand-receptor dissociation may be why insulin, IGF-II and vitamin A did not antagonize the growth inhibitory effects of TMA. Cellular receptors for insulin and IGF-II are well characterized and there is increasing evidence that vitamin A (bound to retinol binding protein) uptake is also mediated by receptors (Blomhoff *et al.*, 1991). Lysosomal processing of the insulin and IGF-II

ligand-receptor complexes is necessary for dissociation (Levy and Olefsky, 1987; Auletta *et al.*, 1992). Monomethylamine is known to inhibit DNA synthesis stimulated by EGF, insulin and serum in rat hepatocytes (King *et al.*, 1981), although, at the concentrations tested, lysosomal degradation was also inhibited. An inhibition of lysosomal degradation prevents growth factor-receptor dissociation, a process which is necessary for stimulation of DNA synthesis.

A deficiency of vitamin A or zinc is known to be associated with growth retardation (see Wasserman and Corradino, 1971; Favier, 1992). Zinc is involved in the expression and regulation of many genes via its role in zinc finger proteins; it is essential for the activity of DNA and RNA polymerase (see Chesters, 1991). In addition, zinc has been shown to have a protective effect against toxins, possibly by gating pores in the membrane (Pasternak, 1988). Zinc was first documented to antagonize the teratogenic action of cadmium in mice in 1965 (Chiquoine, 1965). Cadmium and antivitelline yolk sac antibody inhibit fluid-phase pinocytosis and zinc has been shown to completely antagonize both (Record *et al.*, 1982; Marlow and Freeman, 1987). The lack of a significant antagonistic action of zinc on the inhibition of yolk sac function by TMA supports the view that TMA is inhibiting receptor-mediated uptake.

It is interesting to note that in embryos cultured in serum containing ^{3}H-leucine-labelled proteins, inhibition of the incorporation of ^{3}H-leucine into protein was greater in the embryo (Table 16) than the yolk sac (Table 17). This is likely to be a reflection of the extensive synthesis occurring in the embryo as differentiation takes place. The yolk sac is also growing and synthesizing protein during organogenesis, but at a much reduced rate in comparison to the embryo. Consequently, the embryo is more susceptible than the yolk sac to a reduction in the flow of nutrients induced by TMA, as demonstrated by a greater reduction

in incorporation of ^{3}H-leucine in embryos than in yolk sacs, compared to controls.

The lack of effect of TMA on uptake and incorporation of amino acids into protein in limb buds suggests that the inhibition of yolk sac endocytosis was the limiting step in the embryotoxicity of TMA. This is supported by the fact that the relative proportion of radioactivity (whether from free amino acids or from protein) that was incorporated into protein in embryos and yolk sacs was unaffected by TMA. In other words, once the limiting step is accounted for, the amount of ^{3}H-leucine taken up is as efficiently incorporated in TMA-treated embryos as in controls.

The inhibition of ^{14}C-sucrose uptake in SMG-treated yolk sacs indicates that SMG is interfering in the constitutive activity of the yolk sac, probably at the membrane level. This conjecture is supported by several lines of evidence. Data obtained from incubations of cell cultures with SMG or SMC, the cysteine conjugate of methyl isocyanate, indicate that cytotoxicity is the result of damage to cell membranes (Han *et al.*, 1990). Membrane damage also occurs in cells cultured in the presence of the structurally similar glutathione conjugates of allyl and benzyl isothiocyanates, which are known to release free isothiocyanates under physiological conditions (Bruggeman *et al.*, 1986, 1988). Furthermore, methyl ester derivatives of SMG and SMC, which would be expected to enter cells much more readily than the parent conjugates, did not exhibit a marked difference in toxicity to cell cultures than did SMG or SMC (Han *et al.*, 1990). SMG can release free MIC under conditions of physiological pH and temperature (Pearson *et al.*, 1990). Conjugation of sulphydryl groups in the membrane by released MIC could result in interference of pinocytotic activity.

The SMG-induced inhibition of ^{125}I-BSA uptake by yolk sacs suggests that it also affects specialized transport processes. This inference is supported by the observation that SMG also inhibited the uptake of ^{3}H-leucine (supplied either as ^{3}H-leucine-labelled protein or as

free ^{3}H-leucine) and its incorporation into embryonic proteins. However, the fact that fluid-phase pinocytosis is inhibited to a relatively greater extent than receptor-mediated pinocytosis suggests that SMG may be acting as a general toxin to disrupt pinocytically active membranes rather than to selectively interfere with uptake mechanisms. Covalent binding of MIC, via carbamylation of membrane-associated proteins and alteration of protein structure, is likely to not only inhibit fluid-phase pinocytosis but also to disrupt receptor-mediated uptake. MIC carbamylates a leucine residue in haemoglobin to effect its antisickling property by modifying structure (Lee, 1976). This reaction is irreversible.

Whether ^{3}H-leucine was supplied as free ^{3}H-leucine or ^{3}H-leucine-labelled proteins, inhibition of uptake and of incorporation by SMG was greater in the embryo than in the yolk sac. This may simply be a reflection of the fact that uptake of precursors by the yolk sac is rate-limiting for macromolecular synthesis in the embryo. It might be expected that the yolk sac would be more susceptible, given that SMG probably does not cross cell membranes and that it causes membrane damage (Han *et al.*, 1990). In fact, there may be more carbamylation of proteins by SMG in the yolk sac than in the embryo. The increased incorporation of ^{3}H-leucine in yolk sacs (Table 19) compared to embryos (Table 18) may reflect repair processes subsequent to the SMG-induced damage or an attempt to increase the capacity of compromised uptake. However, as mentioned above, because of the greater rate of growth and differentiation in the embryo compared to the yolk sac, the former is more sensitive to the TMA-induced inhibition of ^{3}H-leucine uptake and so decreased protein synthesis in the embryo is more likely to be noticeable.

Isocyanates are soft electrophiles and preferentially react with soft nucleophiles, such as glutathione, rather than hard nucleophiles such as water (Han *et al.*, 1989). This implies that it is the formation of MIC-sulphydryl group conjugates rather than MIC degradation

products that results in the observed embryotoxicity. Excess GSH would be expected to scavenge any MIC released from SMG and prevent toxicity. GSH was able to antagonize the inhibition of uptake of ^{125}I-BSA induced by SMG and antagonism of SMG-induced cytotoxicity by excess GSH or cysteine has been documented in cell cultures, although much higher concentrations than were practicable in our embryo cultures were necessary. GSH and NAC at concentrations higher than 1 mM inhibited BSA uptake in isolated yolk sac cultures so that antagonism of the effects of SMG at high concentrations of these compounds could not be evaluated. A concentration of 10 mM GSH was necessary to completely antagonize SMG toxicity in cell cultures (Han *et al.*, 1990).

The role of MIC released from the glutathione conjugate in SMG toxicity is supported by the observation that acivicin and aminooxyacetic acid (AOAA), which would inhibit the production of a reactive intermediate by the cysteine conjugate ß-lyase pathway, did not antagonize SMG. Acivicin inhibits the enzyme gamma-glutamyltranspeptidase which catalyses the formation of a cysteine conjugate from the corresponding glutathione conjugate and AOAA inhibits cysteine conjugate ß-lyase, which catalyses the conversion of the cysteine conjugate to a reactive intermediate. The use of these two inhibitors has been shown to inhibit cell and organ toxicity of glutathione conjugates for which conversion to the cysteine conjugate or to the reactive thiol compound are necessary steps in the expression of toxicity (Elfarra *et al.*, 1986; Mertens *et al.*, 1988).

Although cysteine conjugate ß-lyase is found in the liver and intestinal bacteria, it occurs predominantly in the kidney and, as mentioned above, uptake mechanisms for cysteine conjugates have only been documented in the kidney (Elfarra *et al.*, 1986; Schaeffer and Stevens, 1987; Redegeld *et al.*, 1991). The lack of an antagonistic action of acivicin and AOAA to the inhibition of ^{125}I-BSA uptake by SMG could be due to absence of gamma-

glutamyltranspeptidase and cysteine conjugate ß-lyase in yolk sacs. If these enzymes are absent in the yolk sac, one could not conclude that production of a reactive metabolite via the cysteine conjugate ß-lyase pathway has no role in SMG-induced toxicity.

The inhibition of yolk sac uptake mechanisms by SMG occurred at a concentration that inhibited embryonic growth by 20-30% (Guest *et al.*, 1992). Thus this inhibition could explain the reduced growth observed *in vitro*.

4.3 Conclusions

This study has documented foetal toxicity induced by two metabolites of methyl isocyanate, trimethylamine (TMA) and S-(N-methylcarbamoyl)glutathione (SMG), the glutathione conjugate of MIC. TMA but not SMG was toxic *in vivo*, reducing foetal body weight. TMA was selectively toxic to male offspring of mice receiving TMA during mid-gestation (organogenesis); body weight, organ weights, DNA and protein content of the brain and serum testosterone were reduced. The effect on body weight was evident for up to 14 weeks.

TMA and SMG reduced embryonic growth *in vitro* in a concentration-dependent manner. Both agents were teratogenic; TMA induced neural fold defects in the head region and SMG caused spinal deformities. The reduction in embryonic growth caused by TMA was accompanied by a reduction in macromolecular synthesis and could not be antagonized by sulphydryl groups or ascorbic acid. In contrast to TMA, the reduction in embryonic growth caused by SMG was not accompanied by an increase in mortality. Glutathione but not cysteine antagonized SMG-induced growth reduction.

Both TMA and SMG decreased uptake processes of the yolk sac. TMA inhibited receptor-mediated uptake; SMG inhibited the constitutive pinocytosis of the yolk sac in

addition to inhibiting receptor-mediated uptake. In embryo cultures, uptake and incorporation of ^{3}H-leucine-labelled proteins but not of free ^{3}H-leucine was inhibited by TMA. This inhibition could not be antagonized by zinc. SMG inhibited the uptake and incorporation of both free ^{3}H-leucine and ^{3}H-leucine-labelled proteins. Acivicin and aminooxyacetic acid, agents that inhibit the enzymes which catalyse the degradation of SMG to a cysteine conjugate and to a reactive thiol, did not antagonize the SMG-induced inhibition of protein uptake by yolk sacs. Neither TMA nor SMG inhibited uptake and incorporation of free ^{3}H-leucine by isolated limb buds. These data suggest that the predominant cause of embryonic growth reduction induced by TMA and SMG is an inhibition of uptake mechanisms by the yolk sac placenta.

4.4 Clinical relevance

The exposure of thousands of Bhopal residents to methyl isocyanate gas in 1984 was an accident. The industrial use of MIC is expected to continue but intentional or occupational human exposure is likely to be minimal. However, it is interesting to speculate on the possible role of SMG in the chronic toxicity noted after the accident.

It is very possible that SMG was formed in people exposed to MIC in Bhopal. The existence of SMG was not known at the time of the accident and so no measures of tissue levels were recorded. Should serum samples exist, however, it might be worthwhile to analyse them. It has been reported that the cysteine conjugate of benzyl isothiocyanate occurs in the blood at higher concentrations than other organs, with the exception of the liver and kidney (Brusewitz *et al.*, 1977).

The alveolar fluid of the lung contains glutathione at concentrations up to 140 times that in the blood (Cantin *et al.*, 1987). Since exposure to MIC in Bhopal was by inhalation,

formation of SMG is quite possible. The great majority of the people living within the vicinity of the MIC plant (and therefore the majority of the victims) were of low class, living in less than ideal conditions. The likelihood of malnutrition and poor immune status is high. This is pertinent because conditions such as malnutrition and a depressed immune system can result in depletion of tissue glutathione (Golden, 1992). Thus the relative concentration of GSH in the lung would remain high but tissue GSH may be low, which would favour release of MIC from SMG (Bruggeman *et al.*, 1986). It is also interesting to note that isocyanates inactivate glutathione reductase (Babson and Reed, 1978), which would exacerbate a low glutathione status and potentiate MIC toxicity.

The embryotoxic effects of SMG reported in this study are consistent with the suggestion that the systemic toxicities of MIC observed in exposed survivors of the Bhopal disaster may have been mediated, at least in part, by SMG, acting in the capacity of a vehicle for the transport of MIC *in vivo* (Ferguson *et al.*, 1988; Pearson *et al.*, 1990; Pearson *et al.*, 1991). There is not sufficient pharmacokinetic data on SMG available to permit extrapolation from animal to man, but the relatively high concentrations of SMG needed to induce toxicity would appear unlikely to have been achieved in the Bhopal victims. One may however conclude that SMG could certainly have contributed to the observed reproductive toxicity in women exposed to MIC in Bhopal.

Several disease states result in increased levels of methylamines in the blood and populations of these patients present possible cohorts in which the effects of TMA on human foetal development could be examined. People with the inherited disorder trimethylaminuria demonstrate excessive excretion of TMA after ingestion of foods rich in TMA or choline. TMA imparts a strong, unpleasant odour to the urine, sweat and breath so that dietary modification to limit intake of TMA and precursors is practised. Such therapy effectively

eliminates the odour and the increase in serum TMA in most patients. No data on the reproductive function of trimethylaminuria patients has been published but a retrospective study of four families suggested that pregnancy development was unaffected (R. Ayesh, personal communication).

Chronic renal failure is a clinical condition in which serum TMA is consistently elevated. The serum concentrations of many compounds that are normally excreted are raised to potentially toxic levels in uraemia but the precise nature of causality versus correlation of retained compounds to toxic symptoms is still largely unknown (Bergström and Furst, 1978). Caution must therefore be exercised in attributing specific defects in the uraemic syndrome to any one agent. However, because of the documented toxicity of TMA in uraemic patients (Simenhoff *et al.*, 1977, 1978), it is worthwhile to consider the possible contribution of TMA to various aspects of uraemic pathology.

Of particular relevance is the selective toxicity of TMA to the postnatal growth of male progeny of TMA-treated mice. In both human and rat uraemia there is a dysfunction of the hypothalamic-pituitary axis and a primary testicular defect, with resultant low serum testosterone values, possibly due to a defect in 17ß-hydroxysteroid dehydrogenase (Guevara *et al.*, 1969; Blacker *et al.*, 1991). Although the aetiology of the male-selective postnatal growth reduction in mice observed in this study is unknown, the low serum testosterone levels are consistent with the clinical data.

There are other hormone/growth factor abnormalities in uraemia, including decreased synthesis and degradation of calcitriol (Hsu *et al.*, 1991), decreased LDL receptor binding and function (Shapiro, 1991; Portman *et al.*, 1992), decreased metabolic clearance of growth hormone (Metzger *et al.*, 1993) and insulin resistance (Friedman *et al.*, 1991). Children with chronic renal failure demonstrate decreased linear growth (see Rigden *et al.*, 1990). The

biochemical effects known to be induced by TMA could contribute to these abnormalities. Ammonium chloride has been shown to inhibit LDL receptor recycling (Grant *et al.*, 1990) and based on similar weak base properties, TMA could also inhibit receptor recycling, which could lead to decreased LDL binding and function. A dysfunction of membrane activity (phagocytosis) has been reported in uraemia (Vanholder *et al.*, 1991). The aetiology of these hormone/growth factor dysfunctions has yet to be explained, however, and appears to be complex (see Schaefer *et al.*, 1992).

The development of pregnancy in uraemia is often accompanied by a worsening of kidney function and traditionally, termination of the pregnancy was practised. Pregnancy in uraemia is now recognised as quite possible, with successful births in the majority of patients (Imbasciatti *et al.*, 1986; Hou *et al.*, 1985). There is insufficient data to analyse for differences based on the sex of offspring born to these women but complications of delivery, including premature birth, are common (Imbasciatti *et al.*, 1986; Hou *et al.*, 1985).

Millimolar concentrations of lysosomotropic agents can be achieved *in vivo* under normal physiological conditions (Sando *et al.*, 1979; Shiokawa *et al.*, 1987). A recent report indicates that lysosomal degradation of parathyroid hormone is significantly inhibited at 25 μM ammonium chloride (Yamaguchi *et al.*, 1992). Furthermore, at low concentrations of methylamines (< 3 mM), the concentration ratio of cells to medium can be as high as 17:1 (Ohkuma and Poole, 1981). These data imply that despite the high concentrations under which the biochemical effects of methylamines are studied in isolated organs or cells *in vitro*, these effects may be relevant *in vivo*, both to the physiological state, especially in rapidly growing or differentiating tissues and to the pathological state in certain diseases.

CHAPTER FIVE
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Figure 23. Chemical structures

CH_3NH_2

Methylamine (MMA)

$(CH_3)_2NH$

Dimethylamine (DMA)

$(CH_3)_3N$

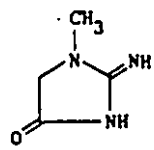
Trimethylamine (TMA)

$(CH_3)_3NO$

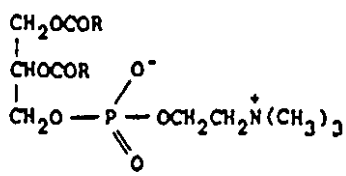
Trimethylamine-N-oxide (TMAO)

CH_3NHCH_2COOH

Sarcosine



Creatinine



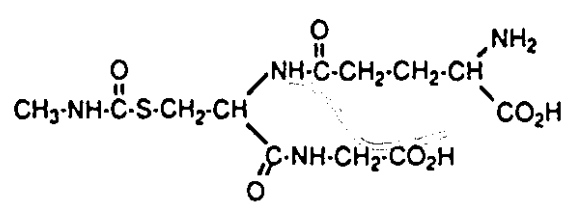
Lecithin

$O=N\text{-}N(CH_3)_2$

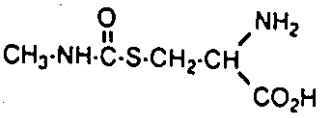
N-Dimethylnitrosamine (NDMA)

$CH_3\text{-}N=C=O$

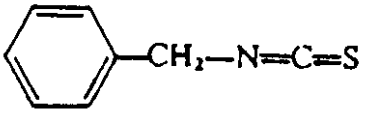
Methyl isocyanate (MIC)



S-(N-methylcarbamoyl)glutathione (SMG)



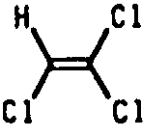
S-(N-methylcarbamoyl)cysteine (SMC)



$CH_2=CH-CH_2-N=C=S$

Allyl isothiocyanate

Benzyl isothiocyanate

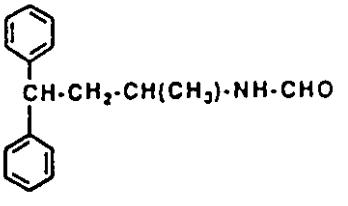


$ClCH_2CH_2CH_2Cl$

1,3-Dichloropropane

Trichloropropene

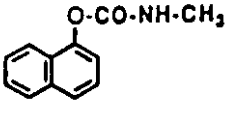
p-Aminophenol(Quinone)

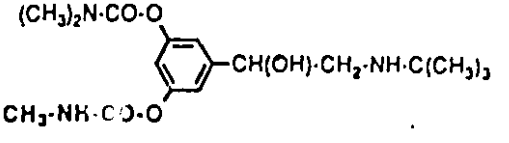


$CH_3-NH-CHO$

N-Methylformamide

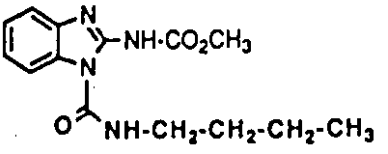
N-Methyl-N-(1-methyl-3,3-diphenylpropyl)formamide



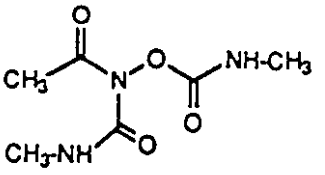


1-Naphthyl-N-methyl carbamate (Carbaryl)

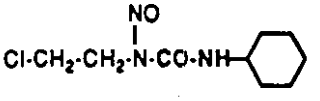
Desmethylbambuterol



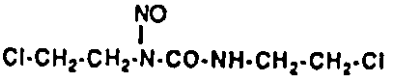
Methyl-1-butylcarbamoyl-2-benzimidazole carbamate (Benomyl)



N,O-*Bis*(N-methylcarbamoyl)acetohydroxylamine (Caracemide)



1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU)



1,3-*Bis*-(2-chloroethyl)-1-nitrosourea (BCNU)

Chlorpropamide