# CHAPTER 1

# INTRODUCTION

Chemicals in embalming solution especially formaldehyde (NIOSH, 2000) have been implicated as a cause of serious health effects. The main risk is presented by exposure to formaldehyde which has been associated with a number of biological effects in humans such as skin sensitization, eye and upper airway irritation (WHO, 2002). Embalming solution used in the Department of Anatomy, Faculty of Medicine, Chiang Mai University contains 14 % of formaldehyde, 28 % of methanol, 2.7 % of phenol, 14 % of glycerol and 34.7 % w/v of potassium nitrate. Formaldehyde is incorporated into embalming solution as fixative, methanol is used as an antipolymerization agent for formaldehyde, phenol is used as a mold preventive, glycerol is a polysaccharide used to help keep the joints of the cadaver flexible and potassium nitrate used as a preservative (http://www.fu-manche.com/morbidaj/embalm.htm). Exposure to harmful chemicals of students might be increasing while dissection of human cadavers due to the fumes from the embalming solution. It is known that embalming solution causes irritation of the skin, nose and eyes of the person who has dissecting especially medical or dentistry students who taking human gross anatomy course. Basing on toxicological and epidemiological evidence, exposure to embalming solution can cause genetic effects such as DNA damage (WHO, 2002; Titenko-Holland *et al.*, 1996; Ying *et al.*, 1997).

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**Formaldehyde**

Formaldehyde ($H_2C{=}O$, Fig. 1) is a flammable, colorless reactive and readily polymerized gas at normal room temperature and pressure, with a relative molecular mass of 30.03 and a pungent odor. Formaldehyde is soluble in water, ethanol and diethyl ether are used in solution or in polymerized form. Under atmospheric condition, formaldehyde is readily photo-oxidized in sunlight to carbon dioxide. It reacts relatively quickly with trace substances and

pollutants in air so that its half-life in urban air, under the influence of sunlight, is short (WHO, 2002).

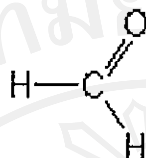


**Figure 1** Formaldehyde structure (WHO,1989).

Absorbed formaldehyde can be oxidized to formate along three different pathways. The overall metabolism of formaldehyde is summarized in Fig. 2.

Formaldehyde reacts virtually instantaneously with primary and secondary amines, thiols, hydroxyls and amides to form methylol derivatives. Formaldehyde is a normal metabolite in mammalian systems. It is rapidly metabolized to formate, which is partially incorporated via normal metabolic pathways into the one-carbon pool of the body or further oxidized to carbon dioxide. Formaldehyde acts as an electrophile and also reacts with proteins and nucleic acids, it reacts with single-strand DNA, but not with double-stranded DNA. This link is reversible. Only formaldehyde crosslinks of DNA and protein are stable. The biological reactions and metabolism of formaldehyde are shown in Fig. 3 (WHO, 2001).

Predominant signs of short-term exposure to formaldehyde in humans are irritation of the eyes, nose and throat, together with concentration-dependent discomfort, lachrymation, sneezing, coughing, nausea, dyspnea and finally death (Table 1)

Formaldehyde is classified as a probable human carcinogen. U.S Environmental Protection Agency (EPA) and The International Agency for Research on Cancer (IARC) identifies it as a class 2A carcinogen, Occupational Safety and Health Administration (OSHA) and National Institute of Occupational Safety and Health (NIOSH) identifies it as a carcinogen. The current OSHA permissible exposure limit is 0.75 ppm (8-hr time weighted average, TWA), 2 ppm for 15 minutes short-term exposure limit (STEL). Other exposure levels such as NIOSH recommended exposure limit (REL) of 0.016 ppm and 0.1 ppm (STEL) (Thrasher, 2001).

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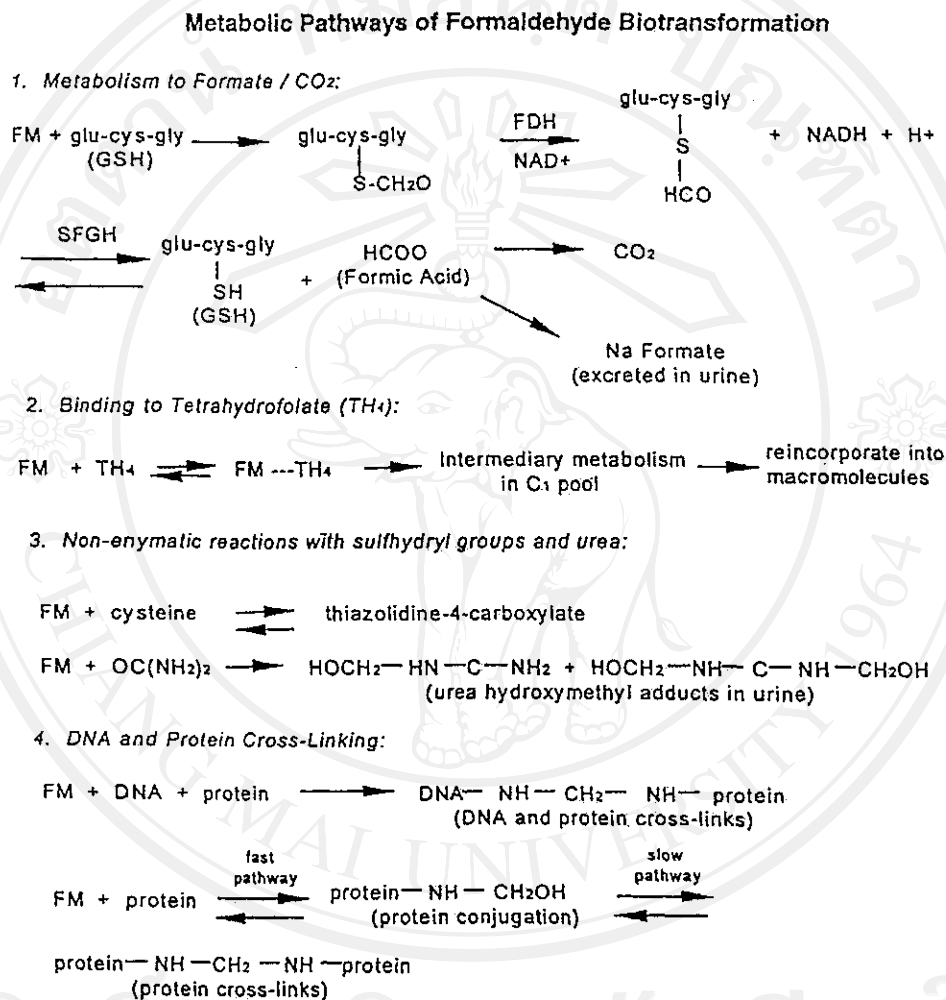


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**Figure 2** Overall metabolism of formaldehyde (WHO, 1989).

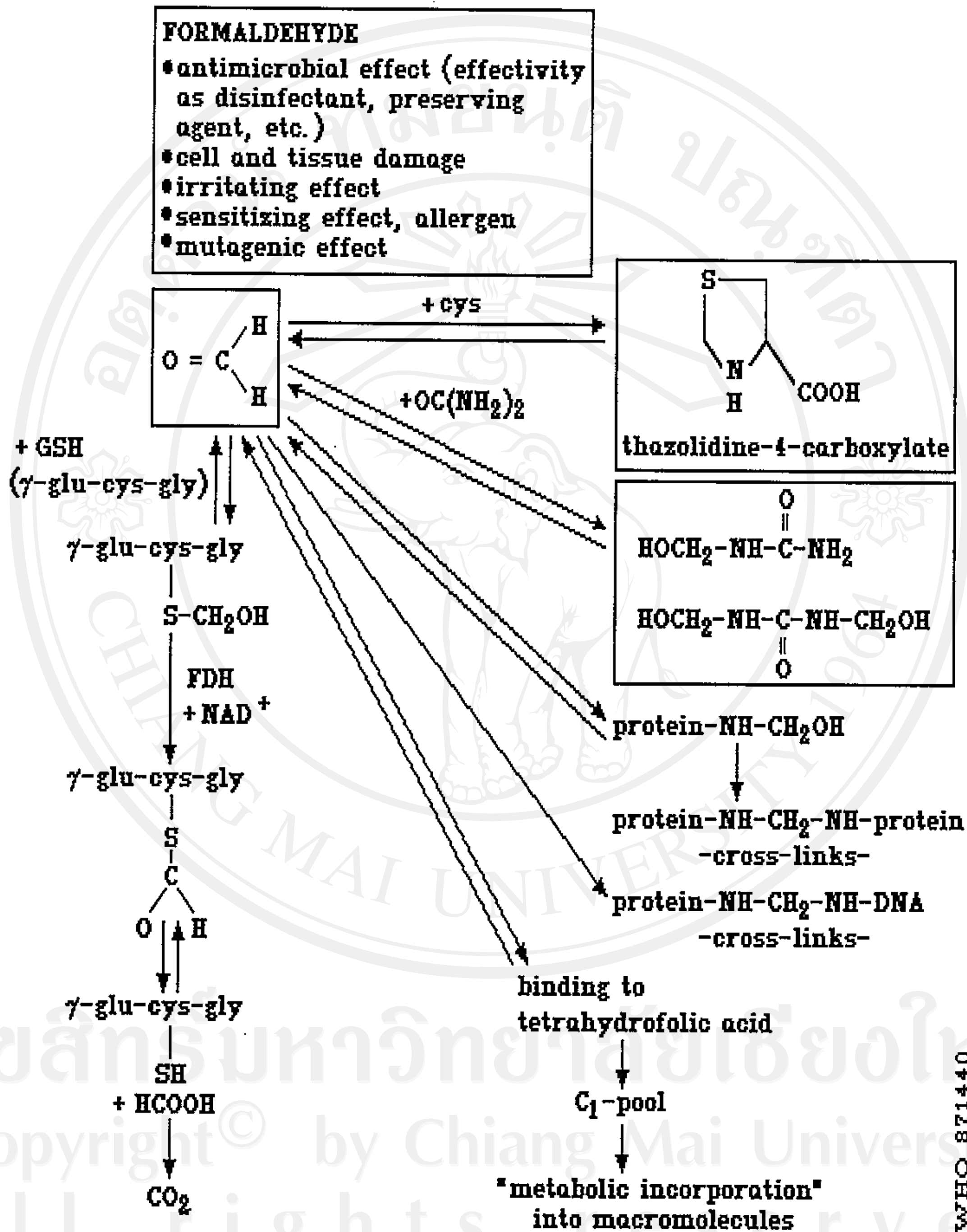


**Figure 3** Biological reactions and metabolism of formaldehyde (WHO, 1989).

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**Table 1** Effects of formaldehyde in humans after short-term exposure (WHO, 2002)

| Concentration range (ppm) | Health effect |
|---|---|
| 0.05-1.00 | Odor threshold |
| 0.01-2.0 | Eye irritation |
| 1.0-3.0 | Irritation of eyes, nose, throat and upper respiratory system |
| 4.0-5.0 | Intolerable |
| 10-20 | Severe respiratory symptoms and difficulty breathing |
| > 50 | Serious respiratory tract injury |
| > 100 | Death |

Formaldehyde induces DNA-protein cross-links in mammalian cells *in vitro* and *in vivo*. The formation of DNA-histone crosslinks proceeds through an initial rapid reaction of formaldehyde with histone followed by conjugation with amino groups of DNA (Quievryn and Zhitkovich, 2000). Formaldehyde produces intra-and inter-molecular crosslinks within proteins and nucleic acids upon absorption at the site of contact (Swenberg *et al.*, 1983). The mechanisms by which formaldehyde induces tumors in the respiratory tract of rats are not fully understood. Inhibition of mucociliary clearance is observed in rats exposed acutely to concentration of formaldehyde greater than 2 ppm (Morgan *et al.*, 1986). There is also evidence that glutathione mediated detoxification of formaldehyde within nasal tissuse becomes saturated in rats at inhalation exposure above 4 ppm (WHO, 2002).

Formaldehyde has long been known to be a mutagenic compound in several biological systems, including bacteria, insects, plant and fungi (WHO, 2002). *In vitro*, it causes increased sister chromatid exchange (SCE) and chromosome aberrations (CA) in CHO cells (Natarajan *et al.*, 1983), CA in human fibroblasts (Levy *et al.*, 1983) and SCE and CA in human peripheral blood lymphocytes (Kreiger and Harry, 1983). In addition there is strong evidence that formaldehyde causes nasal carcinomas in rats (Albert *et al.*, 1982, Sellakumar, 1985). Formaldehyde inhalation increased squamous cell carcinomas of nasal cavity in mice and rats have been observed (Swenberg *et al.*, 1980)

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A sustained increase in nasal epithelial cell regenerative proliferation resulting from cytotoxicity and mutagenicity, for which DNA-protein crosslinks serve as markers of potential, factors contributing to the induction of nasal tumors in rats induced by formaldehyde (WHO, 2002). A sufficient amount of formaldehyde reaching target cells, and saturation of its metabolism to formate can increase the covalent binding of formaldehyde to DNA (Fennell, 1994). Three types of covalent interaction of formaldehyde with DNA have been described (Fennell, 1994).

1 Formation of N-hydroxymethyl adducts with the exocyclic amino groups of adenine, guanine and cytosine.

2 DNA-DNA cross-link formation.

3 DNA-protein cross-link formation.

Formaldehyde is a common component of most embalming solutions utilized to preserve cadavers for subsequent dissection in the gross anatomy laboratory. Student exposure to formaldehyde fume has been previously documented and remains an on-going concern. Yager *et al.* (1986) reported an elevated frequency of sister chromatid exchange in lymphocytes of students exposed to formaldehyde in an anatomy laboratory. Because of its reactivity, inhaled formaldehyde will primarily react with the mucous membranes of the nasal and oral mucosa, respectively dependent on route of inhalation (Hedberg *et al.,* 2000). Human expose to formaldehyde, even considerably below the permissible exposure limit, causes many-fold increased levels of micronuclei and chromosome breakage in oral mucosa (Suruda *et al.*, 1993; Titenko-Holland *et al.*, 1996). The fact that oral mucosa is a target for formaldehyde genotoxicity (Hedberg *et al.*, 2000).

Suruda *et al.* (1993) reported a statistically significant and exposure dependent increase in micronucleus frequency was also found in the buccal cell in 29 mortician students. Formaldehyde exposed calculated as an 8-hr time weighted average were 0.33 ppm on days when embalming were done. Epithelial cells from the buccal area of the mouth showed a 12-fold increase in micronucleus frequency during the study period, from $0.046 \pm 0.17$ per 1,000 cells preexposure to $0.60 \pm 1.27$ per 1,000 cells at the end of the course. Nasal epithelial micronuclei increased 22%, from $0.41 \pm 0.52$ per 1,000 cells to $0.50 \pm 0.67$ per 1,000 cells. In blood cells, the frequency of micronucleated lymphocytes increased 28%, from $4.95 \pm 1.72$ per 1,000 cells to

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6.36 ± 2.03 per 1,000 cells, while sister chromatid exchanges decreased 7.5%. A dose-response relationship was observed between cumulative exposure to formaldehyde and increases in buccal micronuclei in the 22 males subjects.

The frequency of micronucleus in the nasal mucosa of 15 non-smokers exposed to formaldehyde in a plywood factory was found to be 3.5 time higher than in control group (Ballarin *et al.*, 1992). Norppa et al. (1993) reported increased micronucleus in buccal cells of workers in a plywood plant and fiberglass factory, while no change was observed in blood lymphocytes.

Liang *et al.* (1998) found an increased frequency of micronuclei and chromosome aberrations in 13 students exposed to formaldehyde during a 12-week (10 hours per week) of anatomy class. Breathing zone air samples collected during dissection procedures showed a mean concentration of 2.37 ppm. Ying *et al.* (1997) found an increased frequency of micronuclei in nasal and oral exfoliates cells in 25 students in anatomy classes exposed to formaldehyde over an 8-week period. The time- weighted average concentration (TWA) of formaldehyde in anatomical laboratories and in students' dormitories was 0.414 ppm and 0.0098 ppm, respectively. A higher frequency of micronuclei was observed that in nasal and oral exfoliate cells after formaldehyde exposure. No significant increase in the frequency of lymphocyte micronuclei was found after formaldehyde exposure.

**Methanol**

Other potential toxic volatile components of embalming solution include methanol and phenol (Titenko-Holland *et al.*, 1996). Methanol ($CH_3OH$, Fig. 4) is rapidly and well absorbed by inhalation, oral and topical exposure routes (Klaassen *et al.*, 1996). Following absorption the methanol is rapidly distributed to organs according to the distribution of body water.

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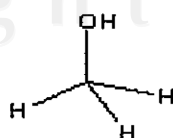


**Figure 4** Methanol structure (WHO, 1997)

Methanol is a clear, colorless, volatile flammable liquid with a mild alcoholic odor when pure. After uptake and distribution, most of the methanol is metabolized in the liver to carbon dioxide. In all mammalian species studied, methanol is metabolized in the liver by sequential oxidative steps to form formaldehyde, formic acid and $CO_2$ (Fig. 5).

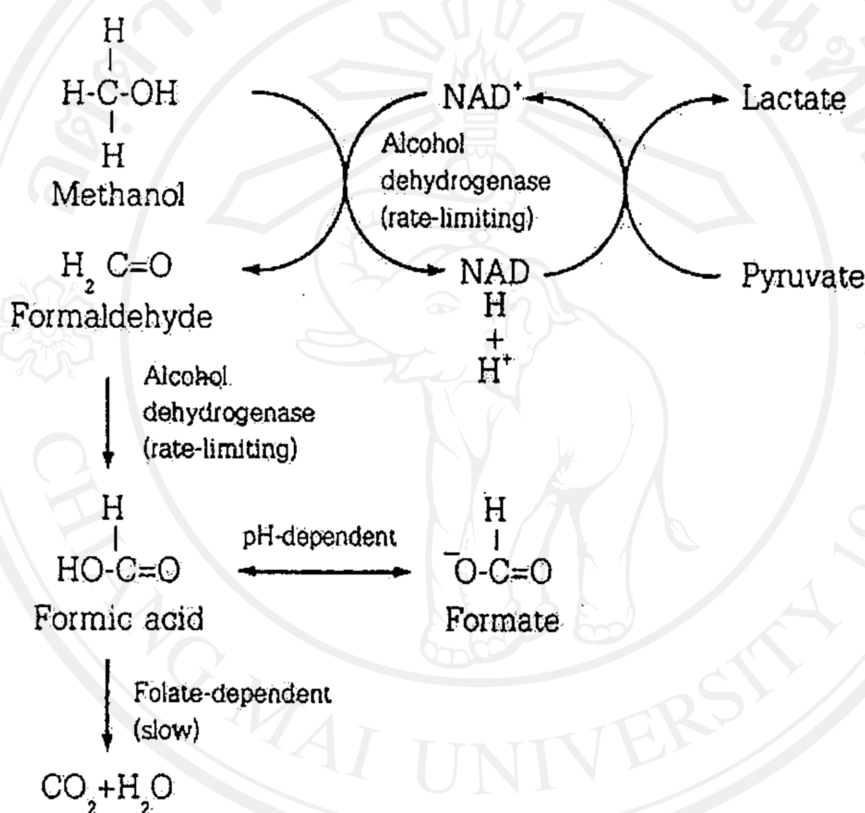


**Figure 5** Scheme for the metabolism of methanol. (WHO, 1997)

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OSHA and NIOSH permissible exposure limit (TWA) and STEL are 200 and 250 ppm, respectively (NIOSH, 2000). Pure methanol has a faintly sweet or slight alcoholic odor, while crude methanol maybe repulsive and pungent. The odor threshold is highly variable depending on purity, estimates of 10 to 20,000 ppm. Exposure for a period of at least one hour to concentrations of methanol in excess, 200 ppm, can cause eye irritation (OEHHA, 2003). Inhalation of acutely toxic concentrations of methanol vapor may cause respiratory failure, rapid respiration, difficulty breathing, abnormally rapid and deep breathing, cessation of breathing and

accumulation of fluid in the lungs. Methanol is slightly irritating to mucous membrane. Inhalation may cause headache, drowsiness, nausea, vomiting, blurred vision, blindness, coma and death (OEHHA, 2003).

Sprague-Dawley rats exposed to 500, 2,000 and 5,000 ppm methanol for 6 h/day, 5 day/week for 4 weeks, exhibited no exposure-related effects except for increased discharges around the nose and eyes which were considered reflective of upper respiratory tract irritation (Andrews *et al.*, 1987). Vendilo *et al.* (1971) reported that rabbits exposed by inhalation to 46.6 ppm methanol for 6 months exhibited ultrastructural change in the photoreceptor cells of the retina and Muller fiber. However, in cynomolgus monkeys, which exposed to 500, 2,000 and 5,000 ppm methanol showed no upper respiratory tract irritation (Andrews *et al.*, 1987). Neither gross microscopic nor ophthalmoscopic examinations disclosed any ocular effects in the monkeys exposed to 5,000 ppm of methanol (Andrews *et al.*, 1987). Although ingestion of methanol historically has been shown to be the most frequent route of poisoning but percutaneously absorption of methanol or inhalation of its vapor is as effective as the oral route in producing methanol toxicity (WHO, 1997)

The structure of methanol (by analogy with ethanol) does not suggest that it would be genotoxic (WHO, 1997). Methanol has given negative results for gene mutation in bacteria and yeast assays. It did not induce SCE in Chinese hamster cells *in vitro* but caused significant increases in mutation frequencies in mouse lymphoma cells. Methanol inhalation did not induce chromosomal damage in mice (WHO, 1997). *In vivo* studies, no increase frequencies of micronuclei in blood cells, of SCE, chromosome aberrations or micronuclei in lung cells were found in mice exposed by inhalation to 1,050 or 5,200 mg/m$^3$ (800 or 4,000 ppm) methanol for 5 days (Campbell *et al.*, 1991). There is no evidence form of animal studies to suggest that methanol is a carcinogen. However in 2001 Gattas *et a.l* (2001) reported that the frequency of oral micronuclei among gasoline station operators in Brazil increased after the introduction of methanol into hydrated ethanol fuel. They concluded that the effect could be related to methanol and possibly also synergistically associated with other components of gasoline.

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**Phenol**

Phenol ($C_6H_6O$, Fig. 6) is absorbed rapidly by all routes. Because of phenol is low volatility, inhalation hazard is limited (NIOSH, 2000). Phenol is a white crystalline solid that melts at 43 $^{o}$C and liquefies upon contact with water. It has a characteristic acrid odor and a sharp burning taste. It is soluble in most organic solvents; its solubility in water is limited at room temperature; above 68 $^{o}$C it is entirely water-soluble. Phenol is moderately volatile at room temperature.

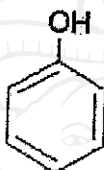


**Figure 6** Structure of phenol (WHO,1994)

After oral uptake of phenol, there is a large first-pass metabolism. It is unclear whether phenol also undergoes first pass pulmonary metabolism, there have been conflicting results (Dickenson and Taylor, 1996). The liver, lung and the gastrointestinal mucosa are the most important sites of phenol metabolism (WHO, 1994). Conjugation with glucuronic acid to phenyl glucuronide and sulphation to phenyl sulphate, have been shown to be major metabolic pathways in several species (Fig. 7). The formation of sulphate and glucuronic metabolites occurs in the hepatocytes, and then transported to the bile or back into the blood (Ballinger *et al.*, 1995). Both *in vivo* and *in vitro* tests have shown covalent binding of phenol to tissue and plasma proteins, some phenol metabolites also bind to proteins (WHO, 1994).

OSHA and NIOSH permissible exposure limit (TWA) and STEL of phenol are 5 and 15.6 ppm, respectively (NIOSH, 2000). Phenol is readily absorbed by the inhalation, oral, and dermal routes. Cellular uptake of phenol is due to its lipophilic character (WHO, 1994). The potential for poisoning through inhalation of phenol vapors has long been recognized, no cases of death following this route of exposure have been report. Symptoms associated with inhalation of phenol included anorexia, weight loss, headache, vertigo, salivation and dark urine (WHO, 1994). The human odor threshold for phenol has reported to range from 0.021 to 20 mg/m$^3$ in air.

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Phenol was classified by US EPA in Group D (data inadequate for evaluating the carcinogenic potential) (WHO, 1994).

As a corrosive substance, phenol denatures proteins and generally acts as a protoplasmic poison. Systemic poisoning can occur after inhalation, skin contact, eye contact, or ingestion. Typically, transient central nervous system (CNS) excitation occurs, then profound CNS depression ensues rapidly. Metabolic acidosis and acute renal failure may complicate the condition. Phenol exposure causes initial blood pressure elevation, then progressively severe hypotension and shock. Atrial and ventricular dysrhythmias have been noted. Mild exposure may cause upper respiratory irritation. With more serious exposure, laryngeal edema, inflammation and ulceration of the trachea, and pulmonary edema can occur. Contact with concentrated phenol solutions can cause severe ocular damage including corneal opacification, if it is, and palpebral bums. When phenol is applied directly to skin, a white pellicle of precipitated protein forms. If phenol is left on the skin, it will penetrate rapidly and lead to necrosis and gangrene. If more than 60 square inches is affected, there is risk of imminent death. Phenol appears to have local anesthetic properties and may cause extensive damage before pain is felt. (WHO, 1994).

The odor threshold of phenol is sufficiently low to provide adequate warning of dangerous concentrations. The potential for phenol to induce micronuclei appears to be related to the route of dosing, with generally positive results when phenol is administered intraperitoneally (Marrazzini *et al.*, 1994) but negative or equivocal results when it is administered orally (Ciranni *et al.*, 1988). This route related difference is likely due to the potential for the first pass detoxification of phenol when it is administered via the oral route (EPA, 2002). Phenol was positive in an *in vitro* assay for the development of micronuclei in human lymphocyte (Yager *et al.*, 1990). Miller *et al.* (1995) also found that phenol was positive in an *in vitro* micronucleus test in CHO cells in the presence or absence of S9 from livers of phenobarbital/betanapthoflavone induced rats, although a stronger response was observed in the presence of S9.

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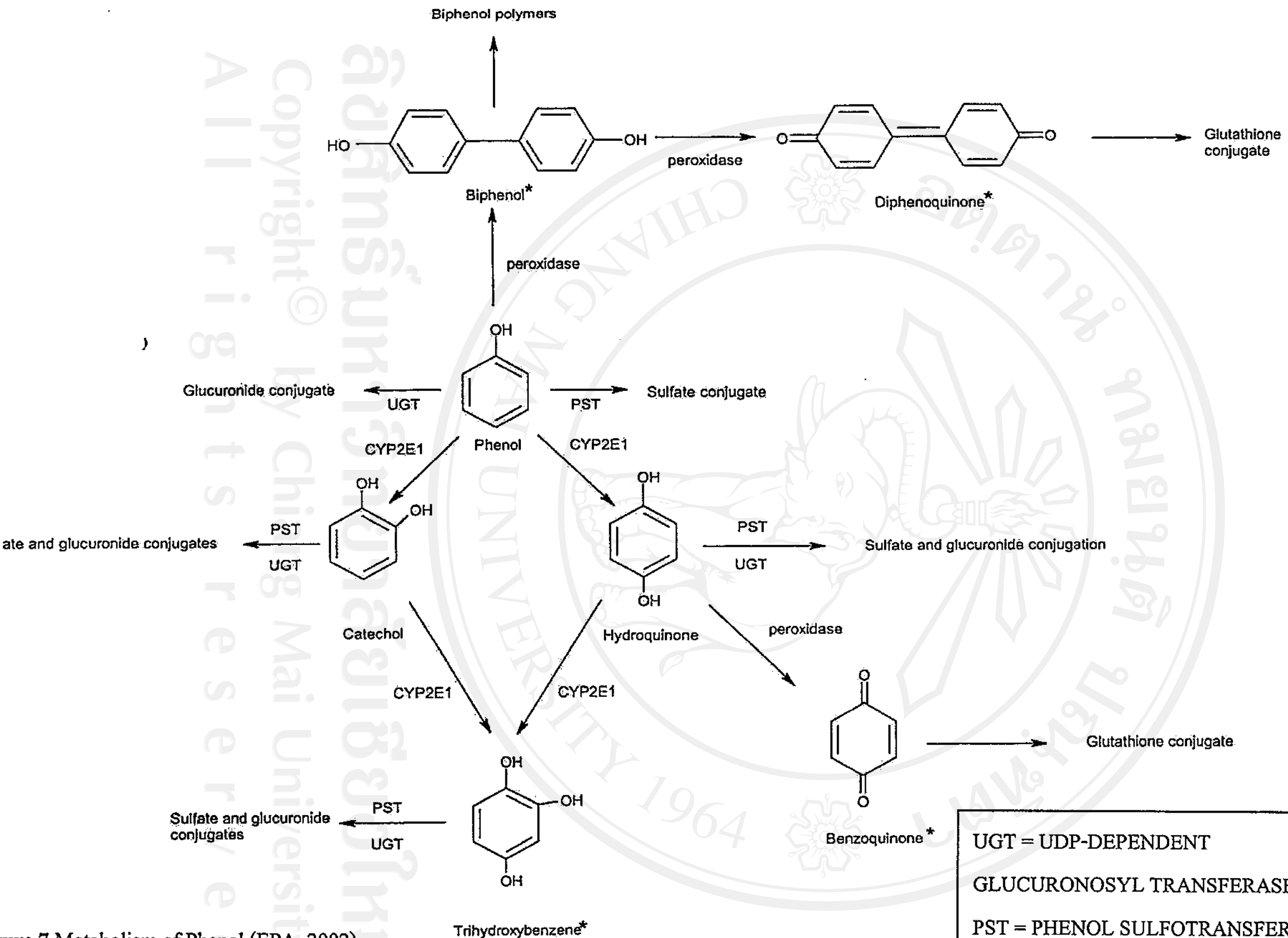


UGT = UDP-DEPENDENT GLUCURONOSYL TRANSFERASE

PST = PHENOL SULFOTRANSFERASE

**Figure 7** Metabolism of Phenol (EPA, 2002)

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**Indoor air sampling**

Analyses of compounds in anatomy laboratory room are not only a measure of indoor contamination but may also provide valuable information for assessment of human indoor exposure. Monitoring methods can involve either active or passive sampling techniques. Active sampling involves using a pump to actively pass air through a sorbent cartridge or collection filter or into an air sample container. Passive sampling of compounds relies on the kinetic energy of gas molecules and diffusion of the gases in an enclosed space onto a sorbent medium. Formaldehyde, methanol and phenol can be collected on adsorbent media by drawing air (at a calibrated flow rate) through a tube containing adsorbent media. At the end of the sampling event, samples are taken to the laboratory for chemical desorption and subsequent HPLC or GC analysis (Office of Research and Standards Department of Environmental Protection, 2002).

Equipments for air sampling include an air sampling pump capable of sampling at the recommended flow rate with the sampling medium in-line, an air flow calibrator, the sorbent sample tubes as specified in the method, the appropriate tube holder or protective tube cover (http://www.skcinc.com/aboror.asp).

Sorbent tube sampling is the NIOSH/OSHA approved method for collecting most hazardous gases and vapors from the air. Federal law has established permissible exposure limits (PELs) for workers' exposures to a variety of airborne chemical hazards. These limits are specified in several ways: 8-hr time-weighted averages (TWAs), short-term exposure limits (STELs) and ceiling values. Eight-hour TWA limits are specified for full-shift exposures. STELs are usually issued as 15 minute exposure limits and ceiling values are issued as peak levels not to be exceeded at any time during the working day. Sorbent tubes have been established as a reliable tool for each of these sampling (http://www.freepumploan.com /pages/1168.html). The sorbent tube is glass with breakable end tips and contains a specially prepared high-activity sorbent. Most tubes have two sections; one for sample collection and the other for backup to assure the complete removal of the chemical hazard from the air sampled (Fig. 8) (http://www.skcinc.com/aboror.asp).

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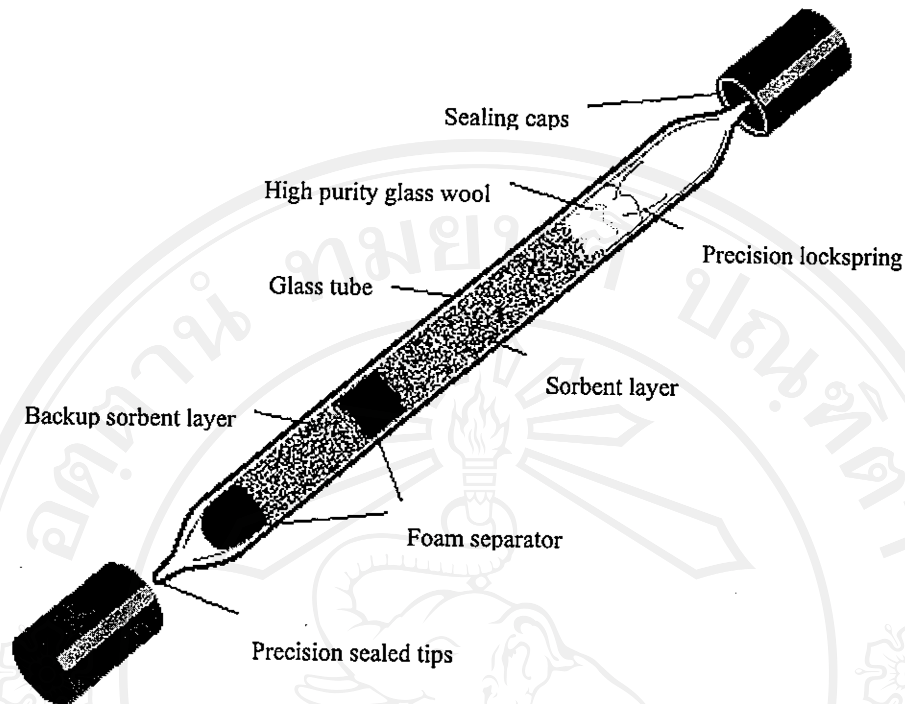


**Figure 8** The sorbent tube (http://www.skcinc.com/aboror.asp)

Active sampling systems, whether internal or external to the suit, draw air through a sorbent at a measured rate. The sorbent removes most (ideally all) of the chemical of concern (Fig. 9). The chemical is then removed from the sorbent by solvent extraction or by heating the sorbent until the chemical is released. A variety of chemical analysis systems can then be used to measure the total amount of chemical desorbed. The total is then divided by the volume of air drawn through the sorbent to approximate the concentration. Because some chemical is usually retained on the sorbent and cannot be measured, chemists usually refine the estimate by calculating the fraction of chemical recovered from sampling tubes that have been spiked with a known amount of the analyte and dividing the quantity observed in samples by that fraction (The National Academy of sciences, 1997).

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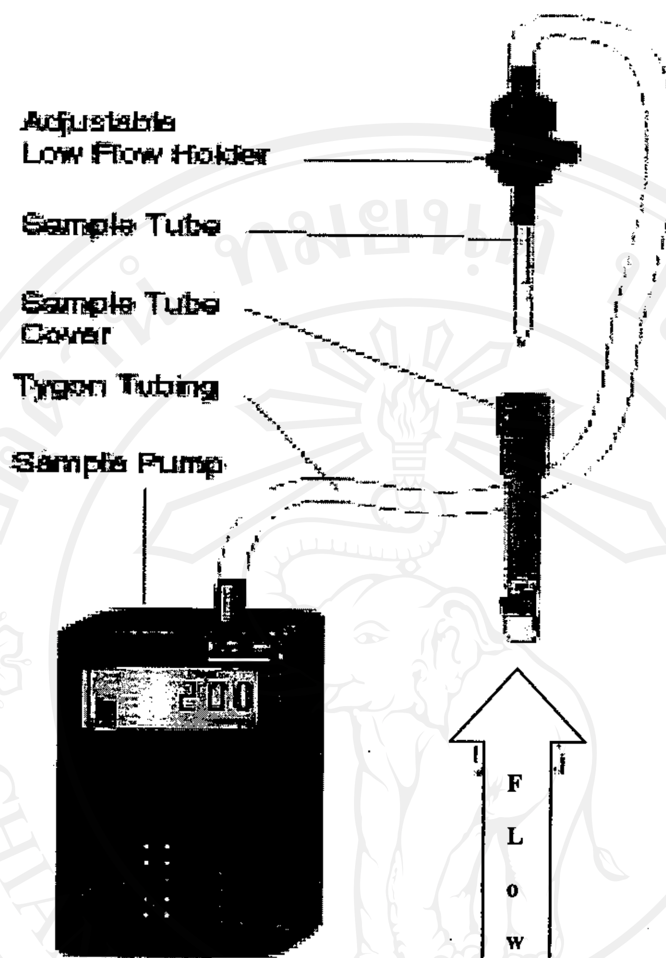


**Figure 9** Pumped sample collection method with adsorbent media tubes (http://www.skcinc.com/aboror.asp)

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**Analysis of air sample**

The analysis of reactive substances poses special challenges for the analytical chemist. An analyte, which is prone to reaction or decomposition during sampling or analysis, has to be determined either by an on-line technique directly in the sample or, in case that this is not available, after chemical derivatization under formation of a more stable product. As short-chain aldehyde is likely to undergo reactions during the analytical process, a large number of dedicated derivatization techniques has evolved in recent year. Some of these are selective for formaldehyde (Vogel *et al.*, 2000).

The importance of derivatizing agents for the analysis of formaldehyde becomes apparent when searching in literature for respective analytical developments and applications. Within the

Chemical Abstracts database, which covers the literature from 1967 until today, more than 1,500 articles are listed which focus on derivatization techniques for the analysis of carbonyl compounds. It is obvious that almost 50 % of all publication are based on the use of 2,4 dinitrophenylhydrazine (DNPH). Even at room temperature formaldehyde reacts rapidly and quantitatively with 2,4-dinitrophenylhydrazine to the low volatile, and stable 2,4-dinitrophenylhydrazone (Sandner *et al.*, 2001). Measurement of the formaldehyde in ambient air was achieved by sampling of air through silica gel held in a holder. The silica gel was coated with trapping solution, DNPH. The reaction with gaseous formaldehyde and DNPH is illustrated in Fig. 10.

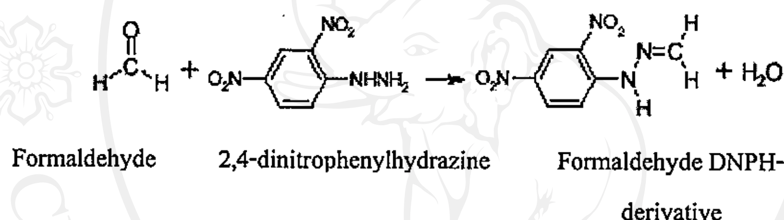


**Figure 10** Reaction between formaldehyde and 2,4-dinitrophenylhydrazine producing 2,4-dinitrophenylhydrazone (Jailson *et al.*, 1999).

A large number of methods for the determination of formaldehyde in air are already known. However, chromatographic methods with UV/visible detection or fluorescence detection are advantageous for the identification of formaldehyde (Buldt *et al.*, 1999). One of the most widely used methods is high-performance liquid chromatography (HPLC) determination using 2,4-dinitrophenylhydrazine (DNPH) as derivatization reagent for formaldehyde, 2,4-dinitrophenylhydrazone (DNPHo) (Buldt *et al.*, 1999). Sampling of formaldehyde in air can be performed using solutions of DNPH in solid sorbents coated with DNPH, including test tubes for pumped sampling. Due to difficulties associated with GC analysis of the DNPHo, specifically related to the low volatility of these compounds, the use of HPLC has been increased (Andrade *et al.*, 1999).

The use of absorption tubes to trap methanol from ambient and workplace air with subsequent liquid or thermal desorption prior to gas chromatographic analysis has been reported

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frequently. NIOSH recommended the use of a glass tube containing two sections of silica gel separated by a portion of urethane foam. Water and isopropanol are used to extract methanol. Analysis of methanol in workplace air has been carried out by headspace GC-FID (WHO, 1997).

Phenol in air samples may be collected by absorption in NaOH solution contained in wash bottles or on filters impregnated with NaOH solution. Phenol in air sample may be collected in tube containing solid sorbent (Tenax, silica gel or carbon). For large air volumes, the NaOH method is usually preferred, whereas for smaller quantities (personal air sampling) solid sorbent tubes have been reported to be more practical. The most important analytical techniques for the detection of phenol are GC-FID and HPLC-UV detection. GC in combination with MS is more sensitive than with FID, but is more expensive (WHO, 1994).

**High performance liquid chromatography (HPLC)** (Lough and Wainer, 1995)

HPLC is a separation technique utilizing differences in distribution of compounds to two phases, called stationary phase and mobile phase. The stationary phase designates a thin layer created on the surface of fine particles and the mobile phase designates the liquid flowing over the particles. Under a certain dynamic condition, each component in a sample has a different distribution equilibrium depending on solubility in the phases and/or molecular size. As a result, the components move at different speeds over the stationary phase and are thereby separated from each other.

This is the principle behind HPLC. The column is a stainless steel (or resin) tube which is packed with spherical solid particles. Mobile phase is constantly fed into the column inlet at a constant rate by a liquid pump. A sample is injected from a sample injector, located near the column inlet. The injected sample enters the column with the mobile phase and the components in the sample migrate through it, passing between the stationary and mobile phases. Compounds move in the column only when is in the mobile phase. Compounds that tend to be distributed in the mobile phase therefore migrate faster through the column while compounds that tend to be distributed in the stationary phase migrate slower. In this way, each component is separated on the column and sequentially elutes from the outlet. Each compound eluting from the column is detected by a detector connected to the outlet of the column.

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When the separation process is monitored by the recorder starting at the time the sample is injected, a graph is obtained. This graph is called a chromatogram. The time required for a compound to elute (called retention time) and the relationship between compound concentration (amount) and peak area depend on the characteristics of the compound. Retention time is therefore used as an index for qualitative determination and peak surface area (or height) as an index for quantitative determination. The retention time of the target compounds and the concentration for each unit of peak area are based on data obtained in advance by analyzing a sample with known quantities of the reference standards. Normally, the reference standards are highly purified target compounds. A HPLC system is basically composed of 1) a pump, 2) an injector, 3) a column, and 4) detector, as shown in Fig. 11.

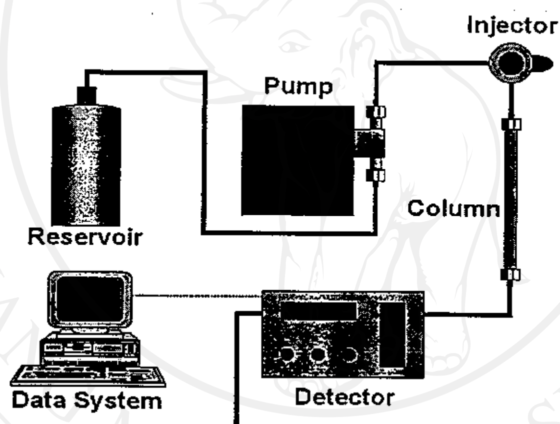


**Figure 11** HPLC system (http://www.lcresources.com/courses/getting_started/1c01.htm).

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## Gas chromatography (GC)

Gas chromatography is a separation method. It involves a sample being vaporized and injected onto the head of the chromatographic column. The sample is transported through the column by the flow of inert, gaseous mobile phase. Two types of GC are encountered which are gas-solid chromatography (GSC) and gas-liquid chromatography (GLC). GLC finds widespread use in all fields of science, where its name is usually shortened to GC. GLC is based upon the partition of the analyte between a gaseous mobile phase and a liquid phase immobilized on the surface of an inert solid. This technique has also gained great interest due to its ease of

automation. Because separation occurs in the gas phase, liquid and solid samples must first be vaporized. Applications of this technique are used in the fields of petrochemistry, pharmaceuticals, flavor analyses and the environmental (Rouessac and Rouessac, 2000).

Chromatography is a separation technique where component molecules (solute) in a sample mixture are transported by a mobile phase over a stationary phase. Mobile phase may be a gas or liquid and stationary phase may be a liquid film on surface of and inert support material or solid surface. Interaction occurs between the solute and stationary phase so that the solute is distributed between the stationary phase and mobile phase. Attraction of the solute for the stationary phase results in retardation of its movement through the chromatography system. Different component (solutes) will move at differing rates since each will have a slightly different affinity for the stationary phase with respect to the mobile phase. Each component or solute (A, B, C) is distributed between the two phases with an equilibrium established defined by the distribution ratio (Rouessac and Rouessac, 2000).

Instrumentation of the gas chromatohraphic system is illustrated in Fig. 12. The basic component of the gas chromatography includes carrier gas, sample introduction, column and detector.

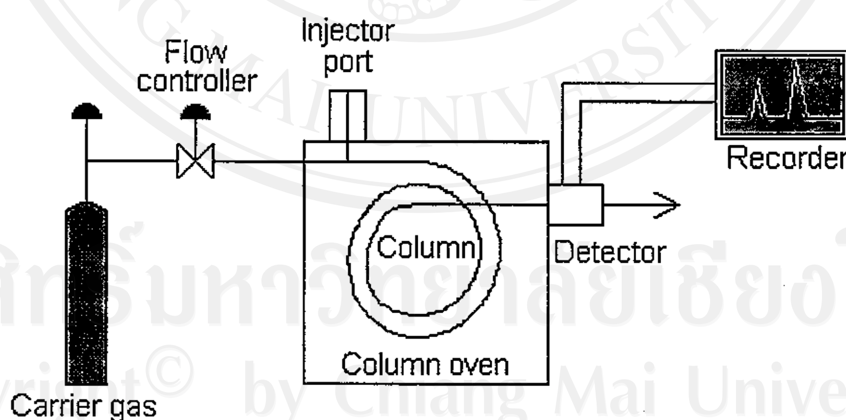


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**Figure 12** Schematic diagram of a gas chromatograph (Srisuwan,2003).

The carrier gas must be chemically inert. Commonly used gases include nitrogen, helium, argon. The choice of carrier gas is often dependent upon the type of detector, which is used. The carrier gas system also contains a molecular sieve to remove water and other

impurities. For optimum column efficiency, the sample should not be too large, and should be introduced onto the column as a "plug" of vapor-slow injection of large samples causes band broadening and loss of resolution. The most common injection method is where a microsyringe is used to inject sample through a rubber septum into a flash vaporizer port at the head of the column. The temperature of the sample port is usually about 50°C higher than the boiling point of the least volatile component of the sample. For packed columns, sample size ranges from tenths of a microliter up to 20 microliters. Capillary columns, on the other hand, need much less sample, typically around $10^{-3}$ μL. For capillary GC, split/splitless injection is used.

The injector can be used in one of two modes; split or splitless. The injector contains a heated chamber containing a glass liner into which the sample is injected through the septum. The carrier gas enters the chamber and can leave by three routes (when the injector is in split mode). The sample vaporizes to form a mixture of carrier gas, vaporized solvent and vaporized solutes. A proportion of this mixture passes onto the column, but most exits through the split outlet. The septum purge outlet prevents septum bleed components from entering the column.

The column oven is used to control the column temperature to a few tenths of a degree for precise work with the aid of a thermostat. The temperature used in separation depends on the analyst's boiling point and the degree of separation required. In the broad boiling point range sample, the temperature program is required to provide reasonable separation and analytical time.

Packed and capillary columns are normally used in GC. The capillary column in preferred for today with its high resolution property. The capillary column is normally produced form fused silica with the length 10-100 m and 0.1-0.53 m internal diameter. The capillary column consists of three types namely, wall coated open tubular (WCOT) support coated open tubular (SCOT) and porous-layer open tubular (PLOT) column. The WCOT is widely used. In WCOT, the liquid stationary phase is coated in the inner wall of column with 0.1-5 μm film thickness. But recently, the bonded phase of which the liquid stationary phase is chemically boned with the signal groups onto the inner wall of the column is widely used. This provides more chemically inert and higher thermal stability. Many types of liquid stationary phase are commercial available (Rouessac and Rouessac, 2000).

The type of detectors that is chosen depends on the compounds of interest and the detection limits required for the analysis. Some detectors are universal. This is because they are

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sensitive to almost every compound that elutes from the column. However, most detectors are sensitive to a particular type of compound. These are called selective detectors. A selective detector is one that can detect only certain compounds, yielding a very simple chromatogram. The ideal determination of an analyte is to have a detector that can detect only this type of analyte. All detectors give a response that is dependent on the concentration of an analyte in the carrier gas. In this work flame ionization detector (FID) was used.

The flame ionization detector (FID) is the most universally used detector in capillary GC analysis because it is sensitive to all organic carbon containing compounds, it has a large linear dynamic range, easy to used, having a first response time and a stable baseline. The gas flow exiting the column passes through a small burner fed by hydrogen and air. This detector essentially destroys the sample. Combustion of the organic compounds flowing through the flame creates charged particle that are responsible for generating a small current between two electrodes. The burner, held at ground potential, acts as one of the electrode. A second annular electrode, called the collector electrode, is kept at a positive voltage and collects the current that is generated. The signal is amplified by an electrometer that generated a measurable voltage. The detector is insensitive toward noncombustible gases such as $H_2O$, $CO_2$, $SO_2$ and $NO_x$. These properties make the FID a most useful general detector for analysis of most organic samples.

The flame ionization detector (FID) is a universal detector and is the most widely used in toxic air analysis as well as other environmental analyses. The FID is considered a universal detector because it will respond to most organic compounds. As the carrier gas elutes from the analytical column into the FID, the eluant is burned in a hydrogen-supported flame and the entire contents of the sample are destroyed. The burning process produces ions that are separated by polarity, collected on an electrode and a current measuring device is used to produce a response. The response gives rise to the typical chromatogram (Clement, 1990).

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**Mass spectrometry (MS)** (Watson, 1985)

Mass spectrometry is an analytical technique that ultimately provides a plot of detector signal intensity (abundance of Ions) versus the mass-to-charge ratio (of ions produced in the instrument; *m/z*). Such a plot is called a mass spectrum and may be considered an "instrumental fingerprint" or exact identifier of a substance. A substance may be identified by a positive

comparison of an experimental mass spectrum with a mass spectrum of a known compound. Large libraries of mass spectra are available. The mass spectrometer is designed to produce ions from a substance, separate the ions according to mass-to-charge ration (*m/z*), and then to detect the number of ions of each different *m/z*.

A substance is introduced in the gas phase into the ion source. There the substance (MH) is bombarded directly or indirectly with electrons interacts in such a way as to produce positive ions, according to the equation:

$$MH + e^{-} \rightarrow MH^{+} + 2e^{-}$$

Ions produced in this way are usually positively changed. The $MH^{+}$ ion has essentially the same mass as the original substance, so it is called the molecular ion. Enough energy is imparted to the substance during ionization that most molecules fragment significantly, producing fragment ions with smaller mass to charge ratio. The mass of the fragment ions and the abundance of ions at each mass vary widely among substances and are unique for a particular compound. Some substances break apart so readily that no molecular ion remains. The ions are accelerated out of the ion source and into the mass analyzer region of the instrument.

An analytical gas chromatograph ultimately produces a plot of detector signal intensity versus time. The plot of peaks versus time describes the elution of sample components and is called a chromatogram. Each peak ideally represents a single, pure compound.

Mass spectrometry, especially when coupled to high-resolution gas chromatography (GC-MS) has certain advantages over other detectors like FID that are commonly used in environmental analysis. One of the most useful features of MS is the generation of a mass spectrum for each individual compound that is unique and serves as a type of fingerprint of each compound. An important feature of an MS detector, is that it is able to discriminate between compound that co-elute during gas chromatography (Office of Research and Standards Department of Environmental Protection, 2002).

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**Solid Phase Microextraction (SPME)**

Solid phase microextraction is a relatively new sample extraction technique that brings some unique capabilities to the chromatographic analysis of dilute solution in difficult matrices, both liquid and gaseous (Hinshaw, 2003). Essentially, SPME has two discrete step: solute

absorption from the sample matrix into a thick, relative to conventional capillary GC columns, layer of silicone or related adsorptive material and transfer of the analytes into a chromatography inlet system by gaseous or liquid means (Hinshaw, 2003). SPME has significant potential to greatly reduce or eliminate solvent consumption and the concomitant issues of used solvent disposal as part of sample preparation.

SPME relies upon the extraction of solutes from a sample into the SPME absorptive layer. After a sampling, during which extraction had ideally reached equilibrium, the adsorbed solutes are transferred with the SPME layer into an inlet system that desorbs the solutes into a gas (for GC) mobile phase. For the extraction step with an externally coated SPME absorptive layer, the layer is exposed to a sample in a liquid or gas phase. The next step after sampling is to transfer the SPME layer and absorbed analyze away from sample exposure and into conditions for desorption in the chromatography mobile phase (Hinshaw, 2003).

The solid phase microextraction process is shown in Fig. 13, 1 cm length of fused silica fiber, coated with a polymer, is bonded to a stainless steel plunger and installed in a holder that look like a modified microliter syringe. The plunger moves the fused silica fiber into and out of a hollow needle. To use the unit, the analyze draws the fiber into the needle, passes the needle through the septum that seals the sample vial, and depressed the plunger, exposing the fiber to sample or the headspace above the sample. Organic analyze adsorb to the coating on the fiber. After adsorption equilibrium is attained, usually in 2 to 30 minutes, the fiber is drawn in to the needle, and the needle is withdrawn form the sample vial. Finally, the needle is introduced into the gas chromatograph injector, where the adsorbed analyze are thermally desorbed by and delivered to the GC column (Sigma-Aldrich, 1998).

SPME is a simple and effective adsorption and desorption technique, which eliminates the need for solvent or complicated apparatus. SPME use for concentrating volatile or nonvolatile compounds in liquid sample or headspace. SPME is compatible with analyte separation and detection by GC or HPLC. By controlling the polarity and thickness of the coating on the fiber, maintaining consistent sampling time and adjusting other extraction parameters, an analyst can ensure highly consistent, quantifiable results for low concentration analytes (Alpendurada, 2000). To date, about 400 articles on SPME have been published in different fields, including environment (water, soil, and air), food, natural products, pharmaceuticals,

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biology, toxicology and forensics. SPME is faster (15 minutes) and much less labor intensive (about 3 minutes of handling time per sample) that liquid-liquid extraction or solid phase extraction, and requires only small amounts of sample and no organic solvent (Sigma-Aldrich, 1998).

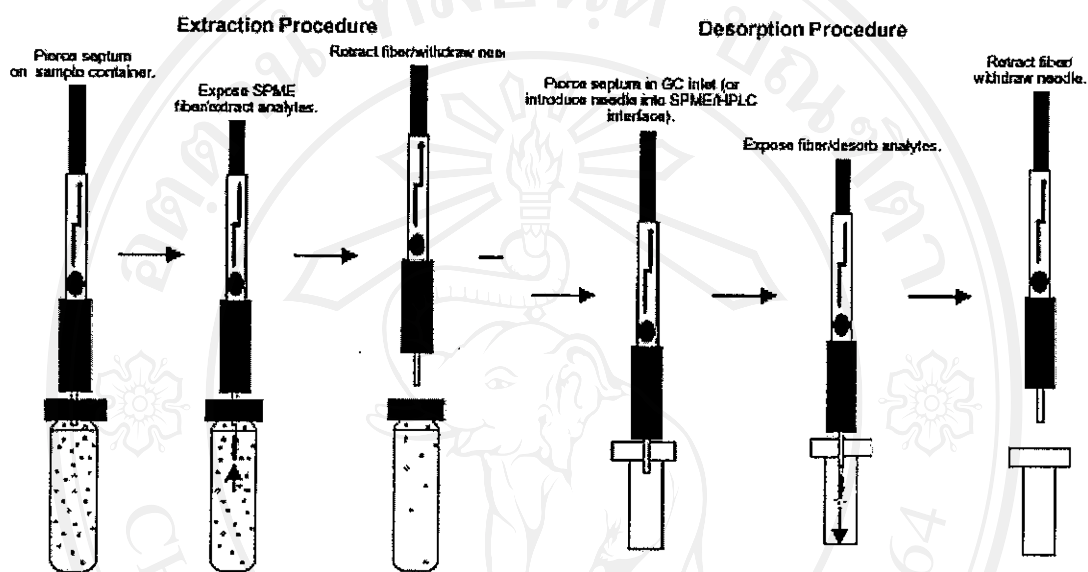


**Figure 13** Solid phase microextraction process (Sigma-Aldrich, 1998).

**Micronucleus**

Micronuclei are intracellular groupings of chromosomal material surrounded by a nuclear membrane, separate from and smaller than the main cell nucleus. Micronuclei chromosomal fragments result, e.g., from: direct DNA breakage, replication on a damaged DNA template and inhibition of DNA synthesis (Albertini *et al.*, 2000).

The micronucleus assay in human is one of the most commonly used methods for measuring DNA damage rates in human populations because it is relatively easier to score micronuclei than CAs (Fenech, 2002). Micronucleus in exfoliated cells emerge during mitosis of the basal layers of the epithelium as extrachromosomal DNA particles when chromosome fragments or whole chromosomes lag behind and fail to be included in the main nuclei of daughter cells. The formation of micronucleus is therefore induced by substances that cause breakage of chromosomes (clastogens) as well as by agent which affect the spindle apparatus (aneugens) pocess of micronucleus induction show in Fig. 14.

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micronucleus expression in a dividing cell

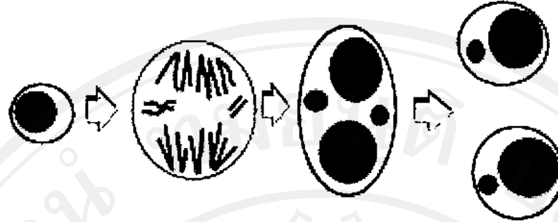

**Figure 14** The origin of micronuclei from lagging whole chromosomes and acentric chromosome fragments in a dividing cell at anaphase (Fenech *et al.*, 1999)

Since 1983, micronucleus formation is a commonly used end point in laboratory models with rodent the micronucleus bone marrow assay with mice has been the most frequently used in vivo assay for routine screening of chemicals. In 1982, Stich and co-workers who used exfoliated cells of the buccal mucosa first described the suitability of the micronucleus test for human biomonitoring studies. Investigations on micronucleus frequencies support the widely accepted assumption that MN is a product of early events in human carcinogenic processes, especially in oral regions (Stich *et al.*, 1982). An increased frequency of micronucleated cells is a biomarker of genotoxic effects that can reflect exposure to agents with clastogenic (chromosome breaking; DNA as target) or aneugenic (aneuploidogenic; effect on chromosome number; mostly non-DNA target) mode of action (Albertini *et al.*, 2000).

Exfoliated cells hold strong potential as a tool for biomonitoring human populations exposed to genotoxic agents or undergoing preventive treatment because they can be easily collected from the mouth by non invasive procedures. In addition, more than 90% of cancer arises in epithelial tissues. Epithelial tissues are in immediate contact with inhaled genotoxic agents (Fenech *et al.*, 1999). Compare to other genotoxicity assays, which are currently used for human biomonitoring, the micronucleus test in exfoliated cells has many advantages (Majer *et al.*, 2001) because it is a simple and fast test system. The cells can be obtained easily and do not have to be cultivated. The process and staining of cells are less time-consuming compared to other test systems and can be performed in laboratories with basic equipment and the end point is well defined and can easily be recognized and the cells can be fixed and stored for long periods of

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time. The simplicity of sample collection with non-invasive method makes the test applicable to large sample sizes (Majer *et al.*, 2001)

This implies that there are no significant differences in micronucleus frequencies between individuals who have been exposed to clastogens for a period of many years and others who were only exposed for a short time. The damage that leads to the formation of micronucleus takes place in the basal layer of the epithelial tissue, where cells undergo mitosis. Rapid turnover of epithelial tissues brings the cells to the surface where they exfoliate. As a consequence, the maximal rate of micronucleus formation in exfoliated cells is seen between one and three weeks after expose to the genotoxic agent (Majer *et al.*, 2001).

**Staining techniques of micronucleus**

**Feulgen-Fast green reaction**

The Feulgen-Fast green staining is a routinely used. The most commonly used staining procedure is the Feulgen reaction for the identification of the DNA of the nucleus and micronucleus, followed by a counterstain with Fast Green to delineate cell cytoplasm. In some studies, Fast Green counterstain was omitted since phase contrast illumination in conjunction with bright field illumination offered and improved discrimination of the texture and micronucleus could be detected easily against a uniform white background. Feulgen and Rossenbeck introduced the Feulgen nuclear reaction for the specific staining of DNA in cytohistochemical sample *in situ* in 1924. This method, HCl hydrolysis, to produce free aldehyde groups in the DNA backbone structure that could be detected by a colored reaction for aldehyde developed 58 year before. This technique has served fundamental objectives in the biological and biomedical sciences concerning location and quantitative distribution of DNA in variety of normal and abnormal cell (Chieco and Derenzini, 1999). During this time, the Feulgen reaction has remained one of the most widely used cytohistochemocal reactions in biology and medicine.

The mechanism of the primary step in the Feulgen reaction, the acid hydrolysis, is straightforward. In a mild acid environment purine bases are detached from the deoxyribose sugar exposing free aldehyde groups and leaving intact the DNA backbone. Therefore, the DNA molecule becomes "apurinic". When it was found that, as hydrolysis proceeds, the DNA filament starts breaking and that fixative and DNA accessibility influence this process. Thus, the

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production of aldehyde groups may be counterbalanced by a loss of DNA fragments from the nucleus that disturb the stoichiometry of the reaction (Chieco and Derenzini, 1999).

Following the hydrolytic step, cells are exposed to the widely used Schiff reagent. Cellular sites with free aldehyde groups in the apurinic DNA molecule bind the bleached pararosaniline dissolved in the Schiff reagent, acquiring a magenta color (Chieco and Derenzini, 1999).

Aldehydes react with Shiff reagent to form a complex, which has a wine-purple color (Fig. 15)

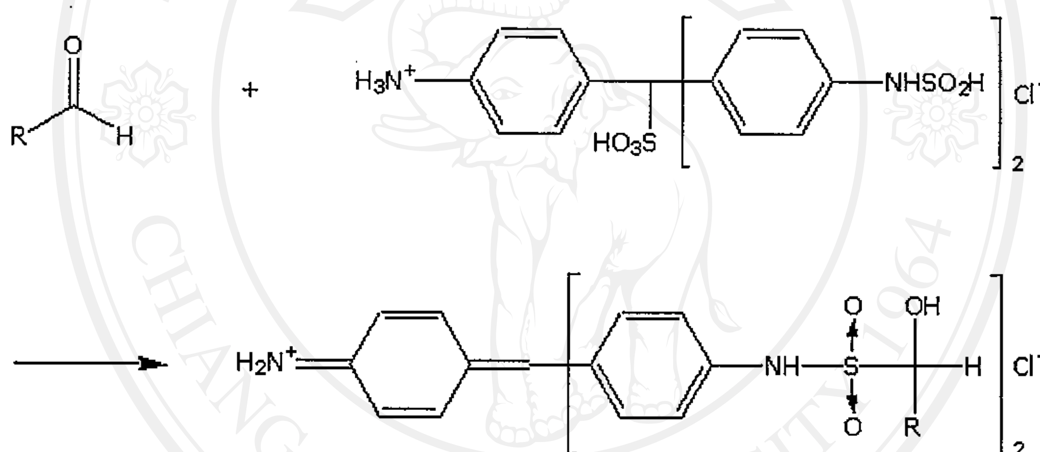


**Figure 15** The Fuchsin-aldehyde reaction (Glaquvich, 2001).

**Florescence in situ hybridization (FISH) technique** (Titenko-Holland *et al.*, 1996)

Florescence in situ hybridization (FISH) technique allows scoring of micronucleus in exfoliated human cells as effectively as standard Feulgen-Fast green staining. The fluorescent dye propidium iodide in antifade solution was used to counterstain the DNA. The main advantage of FISH modification of the micronucleus assay is that it allows for differentiation between two possible means by which a micronucleus can arise. It proved impossible to apply antikinetochore antibody staining which is used in human lymphocytes, in either buccal cells or nasal cells due to the characteristics of membrane in exfoliated cells. FISH staining allowed differentiation of micronuclei that include a whole chromosome, with a centromere, form those containing acentric fragments only.

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Recently, FISH technique with a centromeric probe and propidium iodide as a counterstain was applied to exfoliated human cells. This technique enables to differentation between two possible mechanisms through which micronucleus can arise (clastogenity versus aneuploidy) (Majer *et al.*, 2001). The presence of leukocytes, bacteria and fungi since heavy infections may interfere with scoring. Granulocytes with their segmented nuclei might be mistaken for micronucleated cells if they are not carefully differentiated from epithelial cells. In some case, cells are heavily keratinized and the nuclei fail to stain. Slides with such cells are difficult to evaluate, particularly with the FISH technique, and are therefore discarded (Majer *et al.*, 2001).

**Objective of the present study**

Genotoxicity in the oral mucosa induced by formaldehyde had been well documented in numerous studies (Suruda *et al.*, 1993; Titenko-Holland *et al.*, 1996; Ying *et al.*, 1997; Liang *et al.*, 1998). Three recent studies have addressed the impact of formaldehyde exposure on the micronucleus induction in buccal cells taken from students, who exposed embalming solution (Suruda *et al.*, 1993; Titenko-Holland *et al.*, 1996; Ying *et al.*, 1997). In Thailand, there is no report on the genotoxic effects of exposure to embalming solution. Micronucleus assay was chosen to investigate an increased frequency of micronuclei in buccal cells as a biomarker of genotoxic effects after embalming solution exposure. Micronucleus induction can reflect exposure to agents with clastogenic (chromosome breaking; DNA as target) or aneugenic (aneuploidogenic; effect on chromosome number; mostly non-DNA target) mode of action (Albertini a *et al.*, 2000). The MN assay has been used for screening populations under risk of mutagenic agents that cause oral neoplasias, especially for the identification of pre-clinical steps of the carcinogenic process (Stich and Rosin, 1984). Although inhalation of methanol and phenol has given negative results for micronucleus induction, co-exposure of these compounds from embalming solution may cause additive effect. The hypothesis of this study was embalming solution would cause genotoxic effect indicated as a micronucleus induction. So, the main purpose of this study were:

1) To investigate micronuclei induction in buccal cells collected from dentistry students who exposed to embalming solution vapor at 0, 10 and 15 week-exposure.

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2) To quantify and analyze concentrations of formaldehyde, methanol and phenol in the indoor air of autopsy laboratory room in order to study whether it was over permissible exposure level.

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