# Biological Role of Formaldehyde, and Cycles Related to Methylation, Demethylation, and Formaldehyde Production

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**Abstract:** An overview is given on the analysis, formation, role and occurrence of formaldehyde in living organisms.

Various methods have been used for the determination of formaldehyde in tissues and body fluids. Gas chromatography, thin-layer chromatography and HPLC were employed for the analysis of formaldehyde, mainly after derivatization. The formaldehyde level of human blood and urine was found at the low ppm level. The formaldehyde level could be increased upto several ten $mg/mL^{-1}$ following special dietary supply.

Biochemical pathway of both the formaldehyde production and demethylation/methylation processes is generally connected to the methionine – homocysteine cycles. Another important way of demethylation generated formaldehyde production is given by microsomal cytochrome P-450 dependent oxidation of xenobiotics, such as various drugs prescribed by doctors. Semicarbazide sensitive amine oxidase also produces formaldehyde.

Increased level of formaldehyde may be the indication of either patho-physiological processes, or environmental contamination, or malnutrition.

The formaldehyde-related methylation and demethylation procedures are also detailed. DNA methylation may have an important role in the pathogenesis of certain diseases.

**Keywords**: Formaldehyde, Demethylation, Methylation, Formaldehyde production, Formaldehyde cycle.

## INTRODUCTION

Formaldehyde is a consistent metabolic product of demethylation (both N-demethylation, and O-demethylation, and S-demethylation) [1]. Liberation of formaldehyde by drug demethylation was described almost half a century ago (in 1956) by Axelrod [2]. One-carbon metabolism is important because of the small size of the compounds taking part in it [3]. The carbonyl group of formaldehyde and formic acid is more reactive than that of acetaldehyde and acetate with a factor of about 1000.

Books on biochemistry [3-7] and molecular biology [8] regard S-adenosylmethionine as a key compound of biological methylation. Methyl group acceptors are treated as important compounds. The following examples present certain quintessential substrates and their methylated products (connected with arrows):

- norepinephrine fi epinephrine
- guanidinoacetic acid fi creatinine
- phosphatidylethanolamine fi phosphatidylcholine
- DNA-adenine fi DNA-N-methyladenine
- DNA-cytosine fi DNA-5-methylcytosine
- tRNA base fi methylated tRNA bases
- nicotinamide fi N1-methylnicotinamide
- protein amino acid residues N fi methylated protein amino acid residues

The sites of methylation include amino groups (e.g. arginin and lysine), hydroxyl group (e.g. tyrosine), and residues containing free carboxyl groups.

We can also examine the matter from a different angle: what is the origin of the methyl group. It was clearly stated [4] that "S-methyltetrahydrofolate transfers a methyl group in methionine synthesis, but all other biological methyl transfers involve S-adenosylmethionine".

Tetrahydrofolic acid is the coenzyme, which plays an essential role in the metabolism of one-carbon compounds. The major metabolic procedure of one-carbon group is connected to involvement of the tetrahydrofolic acid part in the 5-methyl tetrahydrofolate, formyl tetrahydrofolate and

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methyl tetrahydrofolate. The one-carbon part is corresponding to methyl-, formaldehyde- and formic acid. This statement is particularly important in the case of certain anticancer drugs. The growth of cancer cells is blocked by the inhibition of DNA synthesis. Fluorouracyl is an analog of uracyl that can be converted into 5-fluorodeoxyuridylate (FdUMP) in the body. FdUMP inactivates thymidylate synthase in an example of suicide inactivation. The reaction is very similar to that of dUMP, except that these two above referenced steps cannot take place, because the proton is replaced with fluoride.

The methyl transfer was recognized in 1950. Challanger [9] wrote up methylation reactions where the methyl source was S-adenosylmethionine. The methionine in mammals is part of the regular diet. Otherwise, in mammals, in certain microorganisms, but not in plants, methionine synthesis takes place by the help of a cofactor derived from vitamin $B_{12}$.

Protein synthesis begins at the N-terminal and it is continued to the C-terminal end of the polypeptide as shown in Fig. (**1**). The AUG initiation codon specifies an amino terminal methionine residue. Although there is only one codon, (5')AUG, all organisms have two tRNAs for methionine, such as $tRNA^{Met}$ and $tRNA^{fMet}$. One of them is used when (5')AUG serves as initiation codon ($tRNA^{fMet}$ or $tRNA_i^{Met}$) and the other one is utilized to code methionine in an internal position of the polypeptide.

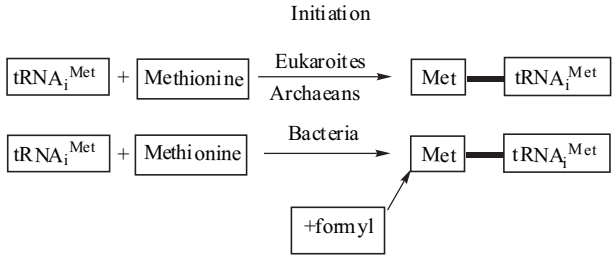


**Fig. (1).** Methionine tRNA is found in cells, and it serves to start protein chains. In bacteria, one formyl group is added, thereby fMet-$tRNA_i^{Met}$ is formed.

In the eukaryotic cells, all polypeptides synthesized by cytosolic ribosomes begin with Met residue rather than fMet. However, a specific initiating tRNA is used that is distinct from the $tRNA^{Met}$ used at (5')AUG codons at interior positions in the mRNA. It is called $tRNA_i^{Met}$ and it can bind at the appropriate site of the small ribosomal subunit (the P site) to begin the synthesis of a protein chain. The other $tRNA^{Met}$ binds only to another ribosomal site, the site A.

## Quantitative Determination of Formaldehyde

Dimedone adducts of formaldehyde are frequently prepared to facilitate quantitative determination of formaldehyde (Fig. (**2**)). Radiometric determination of endogenous formaldehyde was carried out by Szarvas *et al.* [10] using $^{14}C$-labelled dimedone. $^{14}C$-labelled dimedone was synthesized from $^{14}C$-malonic acid in four steps; its specific activity was 954.2 kBq $mg^{-1}$. The formaldehyde level of human blood plasma was 0.4 to 0.6 mg $mL^{-1}$ and human urine contained 2.5 to 4.0 mg $mL^{-1}$ formaldehyde, which was increased to 22.7 mg $mL^{-1}$ after 12 hours following administration of 80 mg hexamethylenetetramine [10].

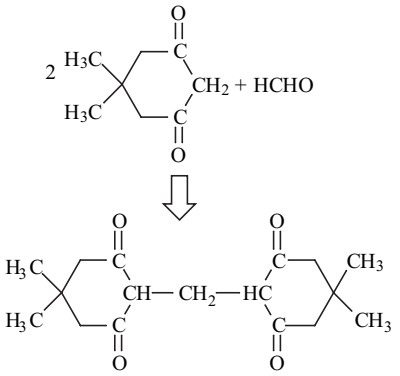


**Fig. (2).** Reaction of formaldehyde (HCHO) with dimedone. Water is also generated during the reaction.

Gas chromatography was an early attempt of formaldehyde determination. Janos *et al.* [11] determined the dimedone derivatives of various aldehydes, such as formaldehyde, acetaldehyde, propionaldehyde and butyraldehyde. The stationary phase was a 1:1 mixture of SE-30 and SP 2401 coated on a 25 m x 0.25 mm I.D. glass capillary column. Structural identification of the peaks was carried out on the basis of their mass spectra using GC-MS with a JMS-O1SG-2 double focusing mass spectrometer.

Formaldehyde determination has also been carried out by liquid chromatography using both thin-layer chromatography and HPLC. Both methods require pre-chromatographic derivatization of the formaldehyde by the use of dimedone, 2,4-dinitrophenylhydrazine (DNPH), or some other reagent. The reaction between formaldehyde and dimedone is given in Fig. (**2**): the formed formaldemethone has an UV absorbance at 254 nm. DNPH-formaldehyde has been usually detected at either 330 or 360 nm.

Both the formaldemethone and the DNPH reacted formaldehyde can be subjected either to TLC or to HPLC. Formaldemethone is usually analyzed by TLC, as TLC silica $F_{254}$ is an excellent stationary phase for its analysis.

Rozylo [12] used dimedone for determination of formaldehyde from teeth in the form of formaldmethone. The teeth material was powdered in three consecutive steps as follow. First, the teeth were treated with liquid nitrogen, then crushed using a hydraulic press, and finally pulverized in a porcelain mortar. Methanolic solution of dimedone was added to the teeth powder, and shaken for about one hour to extract formaldehyde. Centrifugation for about 15 minutes removed the teeth residue. The samples (both the teeth extract, and the standards for formaldemethone) were spotted on silica gel $F_{254}$ plates using a TLC sample applicator, and the plates were tightly covered after sample loading. The plates were developed using a mobile phase consisting of chlorinated hydrocarbons, such as chloroform-dichloromethane, 1:3 (v/v). The formaldemethone gave a

well-defined spot at an $R_F$ value between 0.4 and 0.5, while other substances remained either at or near the start line. The spots of formaldemethone were observable under UV light at 254 nm, while the quantitative evaluation may be carried out using a TLC scanner at 275 nm. The whole experiment should be done with especial care in order to prevent interference from the environmental formaldehyde present in air.

Kalász *et al.* [13] determined formaldemethone originated from the urine of healthy male volunteers. Urine was collected for 24-hour periods, free of any medication other than taking 10 mg of (-)-deprenyl and 10 mg of (+)-deprenyl. Three parallel determinations were carried out using the method of Tyihak *et al.* [14].

Deng and Yu [15] determined formaldehyde from rat urine using HPLC. Other components of the urine were also determined, such as malondialdehyde, acetaldehyde, and methylglyoxal. Samples were collected in the usual way, however, the collected urine was kept frozen using dry ice. Propionaldehyde was selected as the internal standard. Derivatization was carried out using DNPH (2,4-dinitrophenylhydrazine). Formaldehyde-DNPH was determined after extraction with pentane, evaporated into dryness, and reconstructed in water. HPLC was used as the separation technique. The HPLC system consisted of the usual parts, such as an autoinjector, an HPLC pump, column, and a LC spectrophotometer set at 330 nm. The stationary phase was Ultrasphere I.P. (analytical column: 5 mm C18, 250 x 4.6 mm, i.d.), and the mobile phase was 20 mM phosphate buffer at pH 4.6, also containing 32% acetonitrile and 8% 2-methyl-1-propanol, at a flow rate of 1 mL/min. 2-Methyl-1-propanol served as a secondary organic modifier, which significantly increased the retention of the DNPH-aldehyde derivatives. In this way DNPH-formaldehyde and DNPH-acetaldehyde could be separated. A good linear relationship was obtained between concentrations and relative responses over the measured range from 10 through 200 pmol per injection. The correlation coefficient for formaldehyde was 0.99.

Vanhees *et al.* [16] analyzed formaldehyde originated in the reaction between alpha-pinene and hydroxyl radicals. Their study was carried out in a fast-flow reactor equipped with a microwave cavity, and using a high operating pressure. The semivolatile products (such as formaldehyde) were collected in a liquid nitrogen trap coated with a 2,4-dinitrophenylhydrazine (2,4-DNP) solution. HPLC was used for the analysis using benzaldehyde-2,4-DNP and tolualdehyde-2,4-DNP as the internal standards.

Determination of oxo compounds (formaldehyde, acetaldehyde, propionaldehyde, acetone and malonaldehyde) in perfusate served to monitor the oxidative stress in rat heart [17]. The coronary effluents were collected and derivatized with 2,4-dinitrophenylhydrazine. After extraction with pentane, aliquots were separated using HPLC on Ultrasphere C18 column containing 3 mm particles. The mobile phase was acetonitrile-water-acetic acid (400:600:1, v/v/v) and the separation was monitored at 307, 325 and 356 nm. Formaldehyde, acetaldehyde, propionaldehyde, acetone and malonaldehyde were eluted at 6.6, 10.3, 20.5, 16.5 and 5.3 min, respectively.

Formaldehyde was also determined in the form of a fluorescent derivative. Luo *et al.* [18] developed an analytical method for the determination of formaldehyde in human blood plasma, consisting of consecutive steps with preparation of the fluorescent derivative by chemical reaction, clean up and separation. The chemical reaction was carried out at 90 $^{o}$C with ampicillin. The clean up used both precipitation with trichloroacetic acid and extraction with diethyl ether, and evaporation into dryness. The residue was redissolved using a 1:1 mixture of acetonitrile and water. The separation system consisted of an HPLC column, (HP Zorbax StableBond SB-C18m 5 mm, 250 mm x 4.6 mm), the mobile phase was acetonitrile-water (1:3) with a flow rate of 1 mL $min^{-1}$. A fluorescence detector was used at 346 nm and 422 nm, for excitation and emission, respectively. The calibration curve covered a concentration range from 1.6 mg $mL^{-1}$ through 16 mg $mL^{-1}$ of formaldehyde. They found the limit of detection and the limit of quantitation as 0.46 mg $mL^{-1}$ and 0.87 mg $mL^{-1}$, respectively, while the sample requirement was 0.2 mL of blood. They also calculated the intra-day and inter-day recovery and relative standard deviation (RSD), which were over 93% and 98% (recovery), as well as between 3.6-6.2 and 3.1-7.7 (RSD), respectively. Luo *et al.* [18] worked with blood samples spiked with formaldehyde; however, they have not shown any experiments with actual determination of the formaldehyde content of blood samples.

Kobayashi *et al.* [19] used HPLC for the determination of formaldehyde. The procedure consisted of the separation of formaldehyde on a C18 column, postcolumn reaction using Nash reagent at 100 $^{o}$C, and detection by the fluorescence. The intra-day and inter-day variances of the use of the Nash reagent with fluorescent monitoring were less than 10%. This fluorescence monitoring of formaldehyde was about 80-fold more sensitive than using a colorimetric method. Kobayashi *et al.* [19] proved the applicability of their method by following the formaldehyde production by demethylation of aminopyrene, erythromycin, fluoxetine, S-mephenytoin and sertraline in human liver microsomes.

Nakashima *et al.* [20] developed the simultaneous determination of three aliphatic aldehydes (formaldehyde, acetaldehyde and propionaldehyde). They employed derivatization with 4-(N,N-dimethylaminosulphonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD-H). Reversed-phase chromatography with aqueous acetonitrile mobile phase served the separation. The detection was carried out at 560 nm with excitation at 445 nm. Submicromolar level of the aldehydes could be detected. Nebbia *et al.* [21] also determined the formaldehyde with the help of HPLC.

Selected ion flow tube mass spectrometry (SIFT-MS) [2] was used for the quantitative determination of formaldehyde in urine. Similarly, SIFT-chemical ionization-MS was also used to measure the formaldehyde level of cells [23].

Reche *et al.* [24] determined formaldehyde in the finger-paints for children. They used a clean up of formaldehyde with supercritical extraction preceding the chromatographic determination. Formaldehyde was determined using both HPLC with ultraviolet-visible spectrophotometry, and gas chromatography with either flame ionization detection, or

coupled to mass spectrometry. Accuracy and precision in the formaldehyde determination was proven using both samples spiked with formaldehyde, and real samples.

Quantitative analysis of the generated formaldehyde was frequently employed for the determination of the activity of cytochrome P-450 enzyme(s). This type of procedure is especially used at the work with microsomal enzymes. Riley and Howbrook [25] made screened the phenotypes of hepatic Cyt P450-3A function by the analysis of formaldehyde. Their method used N-methyl-$^{14}$C-erythromycin and a reproducible radioassay. The *in vitro* analyses were carried out using pooled human liver microsomes ($K_m$ = 88 mM; $V_{max}$= 345 pmol min$^{-1}$ mg$^{-1}$) and also expressed Cyt P450-3A4 ($K_m$= 33 mM, $V_{max}$= 130 pmol min$^{-1}$ mg$^{-1}$). Bloomer *et al.* [26] determined the activity of human liver P450-1A2 enzyme. N3-Demethylation of caffeine is mediated by Cyt P450-1A2, which is the major pathway of metabolism. Elimination of carbon dioxide is the *in vivo* route; however, *in vitro* reaction produces formaldehyde and formic acid. Using [3-$^{14}$C-methyl]caffeine, a single step of solid-phase extraction was enough for quantification. The $K_m$ and $V_{max}$ values of N3-demethylation was found to be 500 (between 220 and 1200) mM, and 250 (between 115 and 450) pmol min$^{-1}$mg$^{-1}$, respectively.

An indirect method of formaldehyde determination was described by Lizcano *et al.* [27]. Formaldehyde was further oxidized to formate with the reduction of NAD(+) by formaldehyde dehydrogenase. NADH was generated, which can be continuously monitored at 340 nm. The possible interference from the presence of a rotenone-intensive NADH oxidase activity in crude tissue homogenates and microsomal fractions can be essentially minimized by pretreatment of the sample with Triton X-100. An alternative way for the decease of the possible disturbance is the use of APAD(+) instead of NAD(+). In a coupled assay Lizcano *et al.* [27] used their assay to monitor formaldehyde at the oxidative deamination of methylamine by semicarbazide-sensitive amine oxidase (SSAO). The method was suggested to avoid the use of radioactively labeled material ($^{14}$C-methylamine).

Chemical marker of environmental exposure of formaldehyde can also be determined [28]. Formaldehyde (F) binds covalently to human serum albumin (HSA), and a molecular adduct F-HSA is formed. F has the role of a haptene. Formaldehyde exposure is mirrored by the humoral immune response of the serum and anti-F-HSA can be titrated. Two groups of about 90 healthy subjects were examined. Different ratios of F and HSA (10:1 and 5:1) were used and greater sensitivity and specificity was found for the test of 10:1 F-HSA. A significant association in male and female smokers was found with the immunological response obtained with a 10:1 ratio of F-HSA. This type of immunological assay could be useful for public health monitoring of formaldehyde exposure on a large scale.

Electrochromatography is a new and sophisticated method. Capillary electrochromatography was developed for the separation and quantitation of 14 aldehydes and ketones, such as formaldehyde, acetaldehyde, acetone, acrolein, propionaldehyde, butanone, crotonaldehyde, isobutyraldehyde/butyraldehyde, 2-pentanaldehyde, isovaleraldehyde, valeraldehyde, benzaldehyde and hexanaldehyde. The separation system consisted of a volatile buffer (ammonium acetate) which was compatible to mass spectrometry [29]. The mobile phase was methanol-acetonitrile-4mM ammonium acetate adjusted to pH = 8). Electrochromatography was carried out in a fused silica capillary packed with 3 mm octadecyl silica particles. The working range was 0.25 to 79 mg mL$^{-1}$ with a correlation coefficient greater than 0.99. The detection limit was about 0.1 mg mL$^{-1}$ and precision (relative standard deviation, n = 3) was between 2.3 and 9.2%.

## (OXIDATION) HYDROGENATION STATES OF ONE CARBON

Formaldehyde is the key compound in a series of reactions, which involves carbon monoxide, formic acid, formaldehyde and methane, and the oxidation state changes. When the carbon is bonded to the more electronegative oxygen atom, the bonding electron is assigned to the oxygen. Therefore, carbon monoxide, formic acid, formaldehyde, methanol and methane represent 0, 2, 2, 4 and 8 electrons bound to the carbon. When the carbon is subjected to oxidation, it looses the electron. This is the reason that the order of increasing oxidation is alkane fi alcohol fi aldehyde fi carboxylic acid fi carbon dioxide [30].

A special way of forming the formyl group takes place through dioxygenases catalyzed reaction [31]. N-Formylkinurenine is formed from tryptophane on the action of both atoms of the molecular oxygen, and this reaction is catalyzed by tryptophane 2,3-dioxygenase.

Formyl group transfer takes place by the effect of N$^{10}$-formyl H4 folate [32], which plays a basic role in two steps of the *de novo* synthesis of purine nucleotides.

This paper is devoted to the discussion of the sources of the formaldehyde and of the methyl group. This methylation possibility is given by various foreign compounds called xenobiotics. For an alternative route of methylation, the cytochrome P-450 cycle may also be involved. As an intermediate, formaldehyde can also be detected. Sometimes, formaldehyde is not the end product and it may also be mostly eliminated. In any case, the level of formaldehyde is generally well under the usual range. The actual level covers the $10^{-8}$ through $10^{-15}$ mol L$^{-1}$ range, and the analyses are complicated by the possible disturbing contamination from environmental formaldehyde. This is one of the reasons that the importance of formaldehyde in the physiological and patho-physiological processes of the human body has been indicated but the final consequences have still not been settled.

### Formaldehyde Production

Drug-related formaldehyde is generally produced by a hepatic enzyme system called cytochrome P-450 (CYP, Cyt P450). A simplified form of Cyt P450 cycle is given in Fig. (**3**).

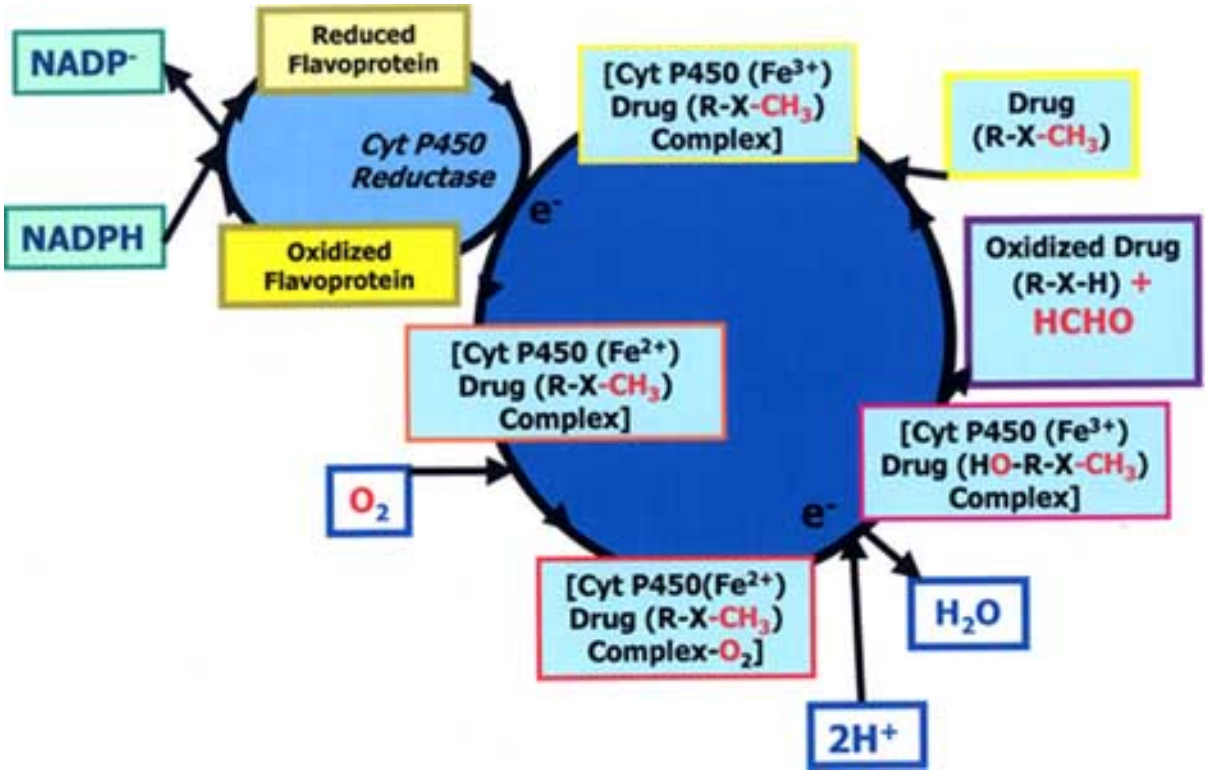


**Fig. (3).** The cytochrome P-450 cycle of methylated drug metabolism.

A wide scale of N-methyl-, O-methyl- and S-methyl-compounds are subjected to demethylation, and thereby formaldehyde is produced. Inhibition of Cyt P450 generally decreases or inhibits demethylation, that is formaldehyde production. On the other hand, induction of Cyt P450 may increase formaldehyde production. A very substantial mass of literature data can be found for the production of formaldehyde by hepatic Cyt P450. Several literature references will be given here.

Meller *et al.* [33] wrote that 5-methyltetrahydrofolic acid, a well-known methyl donor yields formaldehyde by enzymatic degradation. This publication [33] was devoted to correct the earlier published view that 5-methyltetrahydrofolic acid directly methylates dopamine to epinine. The produced formaldehyde performed condensation with dopamine to give a tetrahydroisoquinoline derivative.

There are several ways of formaldehyde production in living organisms, such as demethylation and through special peroxidases, as described by Kedderis *et al.* [34] and Kapoor *et al.* [35]. Formaldehyde, acetaldehyde and malonaldehyde are common products of lipid peroxidation: they are formed in the interactions of polyunsaturated fatty acids and molecular oxygen [36,37]. Oxidative stress can be indicated by lipid peroxidation of polyunsaturated fatty acids. The level of the formation of oxo compounds can be changed [17]. Progressive increase of all five oxo metabolites of lipids (formaldehyde, acetaldehyde, propionaldehyde, acetone and malonaldehyde) was found in the heart tissue perfusate, when the heart was subjected to oxygen-derived free radicals [38]. Hydroxyl radical scavengers, superoxide dismutase plus catalase, definitely influenced the formation of these oxo compounds.

Castonguay *et al.* [38] suggested specific deactivation of tobacco smoke. 4-(Methylnitrosamino)-1-(3-pyridyl)-butanone (NNK) is abundant in tobacco smoke, and it is a potent carcinogen in laboratory animals. Its metabolism can take place in two different ways, such as methyl hydroxylation (leading to formaldehyde formation, an activation pathway) and denitrosation (putative deactivation pathway). (+)-Catechin is a flavonoid being present in tea, wine, apple skin, and 0.1 mM catechin inhibited methyl hydroxylation of 4 mM NNK by 40%.

Formaldehyde rapidly interacts with various cell components, and formaldehyde can also be quickly further metabolized [39]. This is the reason why formaldehyde production is generally monitored *in vitro* (such as using microsomal preparations). Another way of monitoring the formaldehyde is its determination from the various excreta, such as from the urine [13].

## S-Adenosyl-L-Methionine Related Formaldehyde Cycle

S-adenosyl-L-methionine is functioning as a methyl source and also as a donor in a wide variety of living organism [40]. The process may start from serine, uses tetrahydrofolate, homocysteine, methionine, and S-adenosylmethionine as given in Fig. (**4**). The process can be characterized as enzymatic transmethylation reaction, including methylation of either DNA or peptides/proteins.

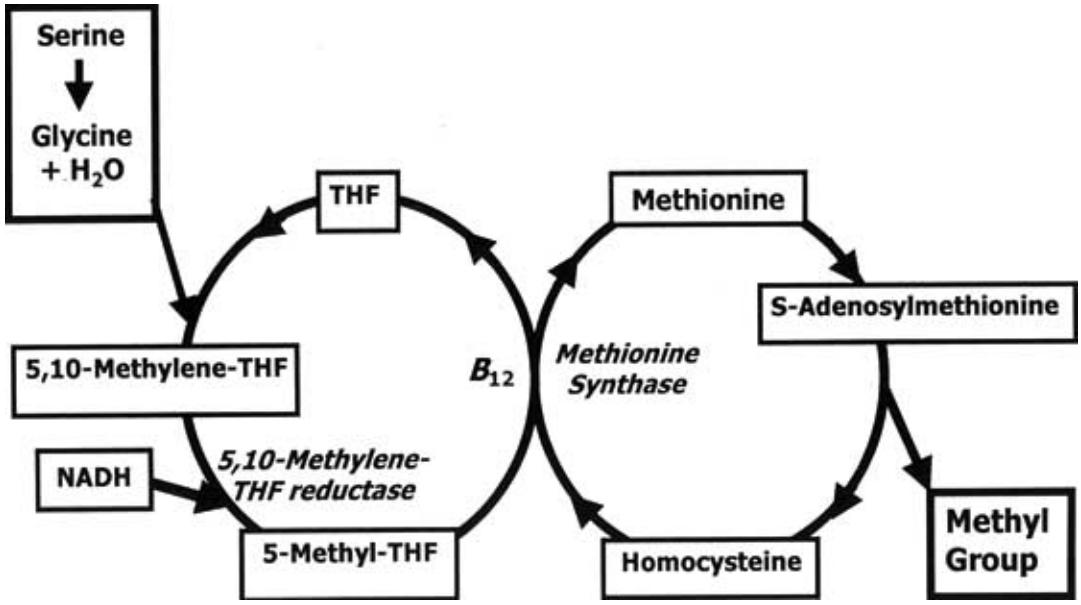


**Fig. (4).** Connection of tetrahydrofolate (THF) and the methionine synthase cycles for generating methyl group from serine.

Tyihak *et al.* [40] rendered direct evidences that neither methyl radical nor methyl cation, but formaldehyde plays a basic role in these processes. They summarized its possible role using the transfer through methionine SAM SAH homo-cysteine in the formaldehyde generation, as shown in Fig. (**5**). More exactly, both the elimination of formaldehyde, and its generation, and also the transmethylation processes can be explained by the existence of such a formaldehyde cycle. Homocysteine takes a methyl group during its transformation to methionine. The source of formaldehyde, that is the origin of the methyl group can be either one of the methyl donors. Such methyl donors are either a betaine, or the usual 5-methyltetrahydrofolate-tetrahydrofolate transfer. This later reaction is supported by

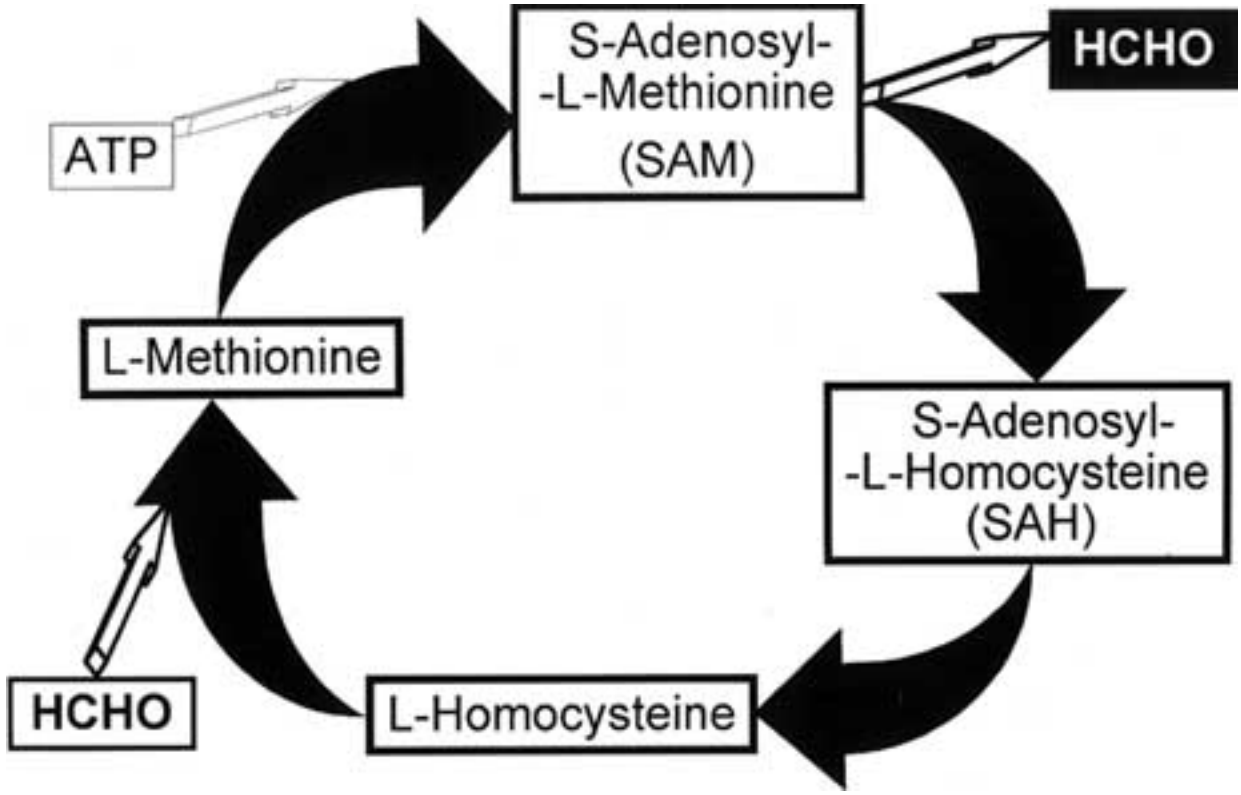


**Fig. (5).** Formaldehyde transfer by the help of homocysteine, methionine, and S-adenosylmethionine. The cycle is based on the publication of Tyihak *et al.* [40].

one of the co-enzymatically active form of vitamin $B_{12}$, (the other co-enzymatically active form of vitamin $B_{12}$ is 5'-adenosylcobolamine). The regulating enzyme, here, is the methionine synthase. The process of L-methionine – S-adenosyl-L-methionine – S-adenosyl-L-homocysteine is generally considered the sole methyl transfer biotransformation; the single exception can be at the synthesis of L-methionine from L-homocysteine [41]. Homocysteine is methylated also through the co-enzymatically active form of vitamin $B_{12}$. Another way is transmethylation by the methyl group of glycine betaine [41]. A further alternative way is postulated by the uptake of formaldehyde.

Experimental results and biochemical arguments support the theory, that a rapid formaldehyde cycle exists [42]. The (formaldehyde) cycle should be able to both eliminate and produce formaldehyde in a wide range of living organisms. The production and elimination of formaldehyde have been widely studied in the cases of various microorganisms, plants, and animals.

An important question is whether formaldehyde is a byproduct of the transmethylation in the biological (physiological) systems, or an intermediary substance of them. Both possibilities can be either supported or denied by the presence of formaldehyde in transmethylation reactions.

## FORMALDEHYDE PRODUCTION BY DRUG METABOLISM

There is an important class of enzymes, which catalyze an overwhelming part of oxidation. This enzyme group has been called either monooxygenases, hydroxylases (as the product is a hydroxyl compound in certain cases), mixed function oxidases, or mixed function oxigenases.

Axelrod [2] described in 1956 that the enzymatic N-demethylation of narcotic drugs liberates formaldehyde. His conclusion was based on the experimental fact, that incubating the drugs with rat and rabbit microsomal preparation yielded formaldehyde.

Formaldehyde can be produced via microsomal cytochrome P-450 dependent oxidation. A wide variety of exogenous compounds having N-methyl (or O-methyl, or S-methyl) group are subjected to oxidative demethylation. The products are the demethylated compounds (the nor-compound) and formaldehyde is also generated. Another way of formaldehyde generation is given by the action of SSAO (semicarbazide-sensitive amine oxidase) which have endogenous substrates such as aminoacetone and methylamine [43-45].

A very special emphasis is caused to the cytochrome P-450 system (P-450) by the metabolic drug interactions [46]. Several specific forms of P-450 enzymes can be involved in the metabolic pathways: these can generally be specified using either inhibitors or molecular expressions or both methods. In the case of methadone, four heterologously expressed P-450 enzymes were able to catalyze its N-demethylation, such as P-450 2C8, P450 2C18, P-450 2D6, and P-450 3A4. However, on the basis of the liver content of these enzymes, Cytochrome P-450 3A4 is the major enzyme involved in the oxidative demethylation of methadone in the average. This is the reason why caution should be advised in the clinical use of methadone together with other drugs that either inhibit or induce Cytochrome P-450 3A4, such as rifampicin, diazepam, etc.

There is an important aspect of cytochrome P-450 catalyzed metabolism. Certain suicide inhibitors can be taken with either the usual drug treatment or with the daily diet. A number of 17-ethynyl-substituted steroids (e.g. gestodene, ethinyl estradiol, levornorgestrol, etc.) irreversibly inactivate human Cyt P450 3A in a time-dependent manner. Moreover, furanocoumarin dimers, GF-I-1 and GF-I-4 are present in grapefruit juice, and they are potent reversible inhibitors of Cyt P450 3A, and also cause time-dependent inactivation [47]. Patients who are subjects to long-term methadone treatment often simultaneously use certain benzodiazepines, such as diazepam [48]. The acute administration of diazepam with methadone doses enhances the effect of methadone, which demonstrates a decrease in methadone biotransformation due to the effect of competitive inhibition with diazepam [49].

Tangeretin was subjected to O-demethylation using both rat and human liver microsome [50]. O-demethylation was proven and followed by the production of formaldehyde. Kinetic study showed a $K_m$ value for tangeretin of about 18 mM. The O-demethylation could be inhibited by carbon monoxide, piperonyl butoxide, 7,8-benzoflavone, propyl gallate, aminobenzothiazole and metyrapone. Rats pretreated either with the usual cytochrome P450 inducers (3-methylcholantrene, phenobarbital, dexamethasone, ciprofibrate) or with flavonoids (flavone, flavanone, quercetin, tangeretine) resulted in increased microsomal O-demethylation of tangeretin only after 3—methylcholantrene and flavone. Tangeretin did not enhance its own metabolism.

There is also indirect evidence that formaldehyde is generated by demethylation. Baek and coworkers [51] investigated the metabolism of DDB (dimethyl-4,4'dimethoxy-5,6,5',6'-dimethylene dioxybiphenyl-2,2'-dicarboxylate). DDB protects the liver against hepatic injury induced by carbon tetrachloride, prednisolone, etc. Moreover, DDB is the most widely used remedy for patients having chronic viral hepatitis in Asia. In the experiment human liver microsome was used, and five metabolites were identified and marked: M1, M2, M3, M4 and M5. M3 and M4 were identified as O-demethylated metabolites of DDB, but the generated formaldehyde was neither identified nor quantitated. At the same time, they identified that the enzyme exclusively involved in the O-demethylation was Cyt P450-1A2.

Baba *et al.* [52] identified a special type of metabolites which have an oxygen on the nitrogen. N-hydroxyamphetamine and N-hydroxymethamphetamine are metabolic intermediates of amphetamine and methamphetamine, and these compounds did not show reactivity towards important endogenous substances, such as

amino acids, peptides, proteins, nucleic acids and fatty acids. On the contrary, these N-oxides reacted with formaldehyde, acetaldehyde, propionaldehyde even at mild conditions, such as pH 7.4 and a temperature of 37 $^{o}$C.

Szatmari [53] assumed that several metabolites of (-)-deprenyl have oxygen attached to nitrogen, such as deprenyl-N-oxide, N-OH-nordeprenyl, N-OH-methamphetamine, N-OH-amphetamine, phenylacetone oxime, N-[(1-methyl-2-phenyl)ethyl]-methanimine N-oxide and N-[(1-methyl-2-phenyl)ethyl]-ethanimine N-oxide. Two of these compounds, deprenyl-N-oxide and N-OH-methamphetamine, are able to generate formaldehyde. The other oxo compounds (phenylacetone and propynylaldehyde) can be subjected to further oxidation. Direct formaldehyde measurements were done using planar chromatography [54].

Nebbia *et al.* [38] studied the oxidative metabolism of monensin, an ionophore antibiotic widely used in veterinary practice as coccidiostatic. The extent of hepatic microsomal metabolism of monensin was based on the determination of formaldehyde, generated by O-demethylation. The turnover number (nmol of metabolized monensin/min nmol cytochrome P-450-1) of the catalytic efficiency (chickcattlepigrathorse) was found to correlate inversely with the interspecies differences in the susceptibility to the toxic effect of the ionophore [21]. The experimental findings of Nebbia *et al.* [21] definitely suggest that formaldehyde production of drug metabolism is far from reaching its toxic levels, if an appropriate caution is taken with the dose.

A simplified reaction of formaldehyde generation has generally been given in the book of pharmacology and related scientific disciplines. The N-methyl compounds are demethylated by the effect of cytochrome P-450-dependent oxidation, and thereby both the nor compound and formaldehyde are yielded. Similarly, the O-methyl- and S-methyl compounds are demethylated via Cyt P450, and formaldehyde as well as the demethylated product are formed.

An increasing pool of evidence verifies that an intermediate N-oxide is formed. This could be the reason that the secondary, tertiary and quaternary amines may produce practically any one of the aldehydes of the former substituents of nitrogen. The intermediate N-oxide did not show any reactivity toward the important components of the body such as proteins, peptides, amino acids, nucleic acids and fatty acids [52]. At the same time, the N-oxide (of methamphetamine) was reacted with a series of various oxo compounds, such as formaldehyde, acetaldehyde, and propionaldehyde. The reaction takes place under mild, nearly physiological conditions.

Cyt P450 3A4 is involved in the dealkylation of buprenorphine, a long acting analgesic of the opiate family [55]. Using human liver microsome, buprenorphine was N-dealkylated with an apparent $K_m$ of 89 $\pm$ 45 mM. The metabolic rate was 3.46 $\pm$ 0.43 nmol/(min x mg protein). The metabolic pathway correlated with the catalytic activity specific to Cyt P450 3A4, and with the immunodetectable Cyt P450 3A4 content of liver microsomal samples. Ten heterologously expressed P450s were tested, and only Cyt P450 3A4 was able to demethylate buprenorphine with a turnover number of 9.6 $min^{-1}$. Moreover, the catalytic activity was inhibited with anti-rat P450 3A4 down to 20% of the control. Buprenorphine metabolism was 62-71% inhibited by three mechanism-based inhibitors (TAO, erythralosamine, gestoden), using nifedipine as competitive inhibitor ($K_i$ = 129 mM) and by 0.6 M ketoconazole (25% residual activity), all these inhibitors are specific to Cyt P450 3A4.

Chronic treatment with amiodarone has double effect on aminopyrine demethylation [56]. Following amiodarone treatment for four weeks, aminopyrine demethylation was significantly impaired, but returned to the pretreatment values in Wistar rats. At the same time, the levels of cytochrome P-450 enzymes were significantly depressed during the treatment and at the very end of the 4 weeks-long treatment. The inhibitory effect may explain the interactions between amiodarone and aminopyrine. The discrepancy between *in vivo* effect and the Cyt P450 level may be explained by the development of a compensatory extra-hepatic site of drug metabolism.

## FORMALDEHYDE CAPTURERS

Besides the formaldehyde generators, there are also formaldehyde capturers. The major formaldehyde capturer is the methionine-homocysteine cycle. S-Adenosyl-L-methionine serves as methyl donor. Various other natural products also act as major formaldehyde capturers [57]. The chemical structure of trans-resveratrol is trans-3,5,4'-trihydroxystilbene belonging to the group of phytoalexins. As a natural product, it is a constituent of grapes and other various medicinal plants. Trans-resveratrol content in the red grape is especially high. The reaction of formaldehyde with trans-resveratrol is considered to be highly beneficial for living organisms, especially for the human being. The elimination of the excess formaldehyde may cause a cardioprotective effect [58-60]. The elimination of formaldehyde from coronaria and from other blood vessels decreases the development of atherosclerotic plaques. Moreover, the product of the fomaldehyde-resveratrol reaction is considered to have cancer preventive activity [61,62].

Tyihak and Szoke [63-65] postulated that certain compounds were able to accept methylation, therefore these compounds capture formaldehyde through enzymatic or chemical reactions.

### Methylation and Formaldehyde Production by $N_5$-Methyltetrahydrofolate

There are evidences that $N_5$-methyltetrahydrofolate [66,67] and $N_5$-$N_{10}$-methylenetetrahydrofolate [68] produces formaldehyde in various living cells. Tetrahydrofolate can bind single-carbon units as the methyl, methylene, and formyl oxidation levels, corresponding to the oxidation level of methanol, formaldehyde, and formic acid, respectively.

The so-called activated methyl cycle [69] serves to give a methyl group to homocysteine, producing methionine. Various conceptions are devoted to the further fate of this methyl group, such as yielding formaldehyde, or to methylate directly a methyl acceptor [69]. The methyl donor is $N_5$-methyltetrahydrofolate, the enzyme is methionine synthase. In the case of bacteria, one form of methyl synthase works with $N_5$-methyltetrahydrofolate methyl

**Table 1. Formaldehyde (as Formaldemethone) Level in Fresh Samples of Various Plants. The Data were Extracted from the Publication of Adrian-Romero *et al.* [70]**

| Species | | Date of Collection | Formaldehyde (mg $g^{-1}$ fresh tissue) |
|---|---|---|---|
| MARINE ALGAE Chlorophyta | Ulva lactuca | November 1997 | 1300 |
| PHAEOPHYTA | Pilayella littoralis | March 1997 | 720 |
| | Fucus spiralis | April 1997 | 1220 |
| RHODOPHYTA | Chondrus crispus | July 1995 | 440 |
| | Porphyra leucosticta | March 1997 | 1350 |
| HEPATICAE | Lunularia cruciata | September 1998 | 80 |
| MACROFUNGI Basidiomycota | Agaricus silvicola | November 1997 | 590 |
| | Amanita muscaria | November 1997 | 170 |
| | Lycoperdon perlatum | November 1997 | 1090 |
| | Coriolus versicolor | January 1998 | 1290 |
| ASCOMYCOTA | Daldinia concentrica | January 1998 | 570 |
| LINCHES | Hypogymnia physodes | December 1997 | 460 |
| | Xanthoria parietina | December 1997 | 380 |
| | Evernia prunastri | January 1998 | 270 |
| | Ramalina siliquosa | January 1998 | 310 |
| | Lichina pygmaea | February 1998 | 1830 |
| TAXALES | Taxus baccata | January 1998 | 260 |
| GNETALES | Ephedra equisetina | November 1997 | 30 |
| PTERIDOPHYTA | Dryopteris felix-mas | November 1997 | 50 |
| | Phyllitis sclopendrium | December 1997 | 310 |
| | Azolla filiculoides | January 1998 | 11 |
| | Doodia maxima | November 1997 | 80 |
| | Ploypodium vulgare | January 1998 | <10 |
| BRYOPHYTA Musci | Thuidium tamariscinum | January 1998 | 130 |
| | Eurhynchium praelongum | February 1998 | 130 |
| | Homalothecium sericeum | February 1998 | 760 |
| | Fontinalis antipyretica | February 1998 | 690 |
| ANGIOSPERMAE DICOTYLEDONES | *Hedera helix* | January 1998 | 310 |
| | Beta vulgaris ssp. maratima | January 1998 | 230 |
| | Rumex sanguineus | January 1998 | 160 |
| ANGIOSPERMAE MONOCOTYLEDONES | Arum maculatum | January 1998 | 1290 |
| | Cordyline australis | April 1998 | 4060 |
| GYMNOSPERMAE GINKGOALES | Ginkgo biloba | November 1997 | 610 |
| GYMNOSPERMAE CONIFERAE | Abies pinsapo | November 1997 | 50 |
| | Sequoiadendron giganteum | November 1997 | 510 |

donor. In mammals and also in bacteria, another form of the enzyme uses either $N_5$-methyltetrahydrofolate or methylcobalamine derived from coenzyme $B_{12}$.

## THE PRESENCE OF FORMALDEHYDE IN PLANTS

Blunden *et al.* [70,71] analyzed the formaldehyde production in a wide range of plants. They detected formaldehyde in the extracts of all tested species of marine algae, macrofungi, lichens, bryophytes, pteridophytes, gymnosperms and angiosperms. The formaldehyde content of the extract was reacted with dimedone to form formaldemethone, and the determinations were carried out using both thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). The results indicate a definite formaldehyde content, as given in Table (**1**).

Katay *et al.* [72] determined endogenous formaldehyde, carnitine, choline, trimethyl-L-lysine and various betaines in macroscopic fungi. The analysis was carried out using overpressured layer chromatography (OPLC), a special type of forced-flow thin-layer chromatography [73]. Both the formaldehyde and the potential formaldehyde generators were identified using OPLC with the help of standards. The OPLC separation was carried out using 20 x 20 cm TLC silica plates 60 $F_{254}$ of Merck (Darmstadt, Germany) as stationary phase. The mobile phase for formaldemethone analysis was either chloroform or chloroform-dichloromethane (25:60, v/v). For the quantitative determination of choline and betaines, isopropanol—methanol--0.1 M sodium acetate (20:3:30, v/v/v) mobile phase was used. The results of identification were confirmed using both proton magnetic resonance spectroscopy ($^1$H-NMR) and matrix-assisted laser-desorption ionization mass spectrometry (MALDI MS), and also electron impact mass spectrometry (EIMS). The quantitative analysis of formaldehyde was carried out in the form of formaldemethone after the formaldehyde reacted with dimedone. Densitometric determination of the OPLC analyses at 265 nm indicated adequate separation of formaldemethone from the so-called beta-front of the developing solvent. OPLC gave symmetrical and sharp peak for formaldemethone with $H_{obs}$ = 25.3 mm and N = 3230. The regression equation for the quantitative determination of formaldehyde was:

$$Y = 301.829\ x - 10519.3495$$
$$(R = 0.9993,\ SD = 6.7002)$$

This equation is valid in a concentration range of 100 through 1500 ng band$^{-1}$ formaldemethone. Endogenous formaldehyde, glycinebetaine and carnitine content of various macroscopic fungi are given in Table (**2**).

Sardi and Tyihak [74] used thin-layer chromatography (TLC), gas chromatography (GC) and HPLC to determine formaldehyde and fully N-methylated substances in different parts of watermelon (*Citrullus vulgaris* L.). The formaldehyde level was found between 0.66 to 173.44 μmol g$^{-1}$ dry material, in the various stages of seed (resting seed, hulled seed after soaking for 24 h, shoot of seedling) and in the leaf (primary leaf, seed leaf of one leaf plant, root of one leaf plant). The results of the determinations highly depended on the excess of the dimedone reagent. Sardi and Tyihak [74] explained the uncertain results with the reaction of the excess dimedone and the N-methylated compounds. The determination involves the reaction of dimedone with the continuously formed formaldehyde on the effect of peroxidases and other demethylases [74].

Kiraly-Veghely *et al.* [75] analyzed the formaldehyde content in parts of white and blue grape berries. The formaldehyde content was determined in the form of formaldemethone using TLC. The level of formaldehyde covered a range between 1 and 7 μg g$^{-1}$ fresh tissue of skin, stem, seed and pulp of Kekfrankos (blue variety) and Chardonnay (white variety) grapes.

Nosticzius [76] demonstrated that formaldehyde is synthesized in the bean leaves in the presence of $CO_2$. The maximum of formaldehyde (and glyoxylate) formation was taking place at pH = 7.

The process of formaldehyde formation is reversible. The direction depends on the existence of light/dark periods. The

**Table 2. The Formaldehyde, Glycinebetaine and Carnitine Content of Certain Macroscopic Fungi. The Data were Extracted from the Publication of Blunden et al. [55]**

| Species | Formaldehyde | | Glycinebetaine | Carnitine | |
|---|---|---|---|---|---|
| | Content [mg g$^{-1}$] (mean value) | SD | Presence (+) or absence (-) in detectable amount | Content [mg g$^{-1}$] (mean value) | *SD* |
| *Agaricus silvicola* | 55 | 4.2 | + | 140 | 10.9 |
| *Amanita muscaria* | 52 | 4.2 | - | 301 | 29.0 |
| *Hygrocybe obrussea* | 68 | 3.9 | - | 630 | 52.9 |
| *Lactarius blennius* | 60 | 4.6 | - | 57 | 6.5 |
| *Oudemansiella mucida* | 40 | 6.6 | - | + | - |
| *Russula atropurpurea* | 39 | 3.2 | - | 145 | 11.7 |
| *Stereum hirsutum* | 32 | 3.0 | + | 233 | 21.0 |
| *Coprinus micaceus* | 45 | 5.0 | - | 619 | 43.9 |
| *Daldinia concentrica* | 23 | 2.8 | + | + | - |

amount of formaldehyde is increased on the effect of illumination. On the other hand, the level of formaldehyde is decreased in dark in the presence of 5 mM $HCO_3^-$, with the parallel increase of glyoxalate, glycine and serine content.

Tyihak and Szoke [65] traced the change in the formaldehyde level of the tissue culture of *Datura innoxia*. The level of formaldehyde had a maximum, and also an increasing period in callus cultures of *Datura innoxia* Mill. through a six weeks period. The (first) maximum was found after the first week in the young tissue and it was devoted to the methylation processes. The change in the trimethyl-lysine content showed certain similarity to that of formaldehyde. The initial high level of formaldehyde was supposed to represent excited formaldehyde which is a reactive compound, and it is rich in energy.

## FORMALDEHYDE IN OTHER LIVING ORGANISMS THAN PLANTS

Formaldehyde is a ubiquitous compound, which is present in all cells [77]. At the same time, formaldehyde is considered as either an initiator or a marker of seriously pathogenic processes, such as carcinogenesis [78-80] and atherogenesis [81,82]. In general, the anti-proliferative activity of formaldehyde may cause the damage. However, in the carciogenesis and in the atherogenesis, the antiapoptotic (pro-proliferative) activity of formaldehyde is not defined. This contradiction of the formaldehyde effect can be easily explained by the basic biological rule that an essential part of the physiological and pharmacological effects act on a dose-dependent manner. Both the site, and the level and time course of formaldehyde are definitive in the outcome of its final action. Cell proliferation in a certain compartment of the body can lead to the replacement of the loss of cells, such as counterbalancing the neuro-degeneration, prevention from cell damage caused by the oxidative stress, etc. There is a proven function of the formaldehyde effect to prevent apoptotic processes, which can also be depending on the site of the action.

Formaldehyde is a Janus-faced compound. An increased level of formaldehyde my cause essential increase in cell survival. Other cases mirror toxic symptoms with highly elevated formaldehyde level. Increase of neuronal cell survival is a common "side-effect" of a wide variety of drugs used as monoamine oxidase inhibitors, such as (-)-deprenyl [83] and its N-methyl bearing analogues [84]. On the other side, elevated level of formaldehyde-producing enzymes can add an additional factor to the complications of certain diseases. However, these enzymes yield a couple of other potential cytotoxic metabolites than formaldehyde (e.g. serum semicarbazide-sensitive amine oxidase yields hydrogen peroxide and acrolein in addition to formaldehyde) [85]. This is why an elevated level of certain formaldehyde producing enzymes (not the formaldehyde) is generally considered as either a risk factor or a clinical marker of diseases, such as atherosclerosis [85].

There are several indications on the basic role of formaldehyde in the living organisms. As explained in the book of Voet *et al.* [7] proteins of *E. coli* begin with N-formyl-methionine. Formation of formaldehyde adduct with highly elevated levels of certain exogenous compounds may indicate pathological symptom in rats. Experiments with rats and mice proved that intraperitoneal injection of hydrazine resulted in the methylation of DNA of liver [86]. Liver DNA was isolated and analyzed by HPLC and fluorescence spectrophotometry to monitor the changes. A dose-related (0, 5, 10, 20, 40 mg hydrazine/body weight, i.p.) formation of 7-methylguanine and O6-methylguanine was observed. Various evidences suggest that the reaction takes place as hydrazine reacts with endogenous formaldehyde; a stable intermediate (possibly tertraformyltrisazine) is formed, which is metabolized by alcohol dehydrogenase and aldehyde dehydrogenase, forming an active methylating agent. O6-methylguanine is a promutagenic base, and it has a disappearance half-life of 200 h and 17 h in B6C3F1 mouse liver and Swiss Webster mouse liver DNA, respectively [86].

Hover and Kulkari [87,88] published an alternative way of formaldehyde production. Non-heme iron proteins, such as lipoxygenase (LO) of human term placenta (HTP) and of soy bean (SB) also mediated N-demethylation. Model substrate was chlorpromazine, a prototypic phenothiazine. The reaction depended on the enzyme concentration, substrate concentration, incubation time, and the pH of the medium. Specific activity under optimal assay condition was $1.7 \pm 0.3$ nmol HCHO/(min x mg protein) for HTP-LO mediated demethylation of chlorpromazine. Co-oxidase activity was supplied by tert-butyl hydroperoxide for the demethylation of chlorpromazine. HTP-LO and SB-LO also mediated the demethylation of other phenothiazines and N-N-dimethylaniline (DMA). Specific activity for SB-LO mediated demethylation of DMA was $200 \pm 18$ nmol HCHO/(min x mg protein) or $23 \pm 2$ nmol HCHO/(min x nmol of enzyme). The specific activity for HTP-LO catalyzed demethylation of DMA was $33 \pm 4$ nmol HCHO/(min x mg protein). Formaldehyde production was monitored by the Nash reaction [87]. Nordihydroguaiaretic acid, a classical inhibitor of lipoxygenase, as well as certain antioxidants and free radical scavengers markedly decreased the rate of formaldehyde production. Several other methyl-compounds (such as N-methylaniline, N,N,N',N'-tetramethylbenzidine, N,N-dimethyl-p-phenylenediamine, N,N-dimethyl-3-nitroaniline and N,N-dimethyl-p-toluidine) were also demethylated by SB-LO. The N-oxide intermediate of N,N-dimethylaniline was not detected. The absence of N-oxide can mean an essential difference between N-demethylations catalyzed by SB-LO and Cyt P450 [88].

## FORMALDEHYDE EITHER INHIBITS OR GENERATES APOPTOSIS

Control of the cell proliferation can be modeled by the use of cell cultures. The effect the proliferation of cultured tumor cells can be experimentally investigated employing various doses of formaldehyde. Tyihak *et al.* [89] treated HT-29 human colon carcinoma and HUV-EC-C human endothelial cell cultures with doses of formaldehyde varied between 0.1 and 10.0 mM. Ten mM of formaldehyde resulted in the total kill of both cell types in the cultures. In

the case of HUV-EC-C endothelial cell culture, 1 mM of formaldehyde caused a slight, but non-significant decrease of the cell number. However (on the contrary), a promoting effect was found in the cell proliferation by the presence of 0.1 mM of formaldehyde. Both on the 1st, and the 2nd and the 3rd day the cell number was multiplied by a factor of over 1.5. Tyihak *et al.* [89] also calculated both the mitotic and the apoptotic indices by counting 2000 cells and expressed it in percentage. While 1.0 mM of formaldehyde slightly decreased the mitotic index and increased the apoptotic index, 0.1 mM of formaldehyde increased the mitotic former, and decreased the latter. Their results were confirmed by an analysis of the cells using flow cytometry.

Szende *et al.* [90] described the dose-dependent effect of resveratrol on the proliferation and apoptosis in endothelial cell cultures. As resveratrol is reactive to formaldehyde, the resveratrol effect on the endothelial cell culture is double-related to its formaldehyde capturing effects. The level of formaldehyde is regulated by resveratrol, and the reaction products (of formaldehyde captured by resveratrol) promote activity on apoptosis [90].

## INCREASED FORMALDEHYDE LEVEL AS MARKER OF CERTAIN PATHOLOGICAL CASES

Spanel *et al.* [22] quantified formaldehyde in the urine headspace using analysis by selected ion flow tube mass spectrometry (SIFT-MS). The formaldehyde level was elevated in the headspace of the urine from the cancer patients as compared to that of the healthy controls. SIFT-MS may give a new, non-invasive indicator of the presence of an early-stage of tumors in the body [91].

Elevated plasma semicarbazide-sensitive amine oxidase (SSAO) activity was found by Garpenstrand *et al.* [91] in Type 2 diabetes mellitus complicated by retinopathy. The generated toxic metabolites (such as a formaldehyde) are suspected to develop diabetic retinopathy. A potent and specific inhibitor of human SSAO might help to prevent retinopathy in diabetes mellitus.

The increase of the formaldehyde level may be an indication of pathological process of the teeth, as described by Rozylo *et al.* [12, 92-97]. In the cells the level of formaldehyde [12] indicates the actual stage of physiological and non-physiological processes especially in the human teeth. A high formaldehyde level was found in the extracted deciduous teeth, and in the human teeth of adults with extensive deep carious lesions. Similarly, high formaldehyde level was determined in certain teeth extracted due to periodontal disease, also having some carious lesions. In the group of unerupted teeth, low level of formaldehyde was found. Rozylo *et al.* [97] also found that the determined formaldehyde level was increased in the carietic teeth in comparison with healthy teeth. In the case of parodontic teeth samples, a dramatic increase of formaldehyde was found. Also, the level of betains decreased dramatically. The determinations were carried out using both overpressured layer chromatography (OPLC), and HPLC + MS.

Szende *et al.* [98] described that a measurable elevation in the level of formaldehyde was found in the culture of PC-3 prostate cancer cells. The control meant 104 cells/well, and 100 $\mu g\ mL^{-1}$ methyl-ascorbigen as well as the effect of 100 $\mu g\ mL^{-1}$ dimedone was checked. Formaldehyde level was determined after 48 hours, and the cell proliferation was controlled each day for three days by counting the cell number. When methyl-ascorbigen (indol-containing derivative of the L-ascorbic acid) was added to the prostate cancer cell culture, the formaldehyde level was increased by 64%. At the same time, the number of cells significantly decreased in the presence of methyl-ascorbigen, while the addition of dimedone (formaldehyde capturer) to the culture medium definitely but not significantly increased the number of prostate cancer cells. Another interesting phenomenon was the induction of apoptosis under the effect of methyl-ascorbigen. The morphology of the prostate cancer cells was controlled after 48 hours of the treatment. Neither the control, nor the dimedone-treated cultures were altered notably. However, an alteration subjected almost one half of the methyl-ascorbigen treated cells underwent the process of apoptosis (karyopyknosis, shrinkage of the cytoplasm, Apop-Tag positivity). The ratio of apoptotic to "normal" prostate cancer cells were 1.1 to 98.9, 1.0 to 99 and 45 to 55 in the cases of control, dimedone treated and methyl-ascorbigen treated prostate cancer cell cultures, respectively. Furthermore, the mitotic ratio of the prostate cancer cells were 3.1, 3.2 and 0.6 in the control, in the dimedone treated and in the methyl-ascorbigen treated prostate cancer cell cultures, respectively. The effect of methyl-ascorbigen on the apoptosis could be essentially counterbalanced by the presence of dimedone in addition to methyl-ascorbigen (100 $\mu g\ mL^{-1}$ methyl-ascorbigen and 100 $\mu g\ mL^{-1}$ dimedone in the culture of prostate cancer cells). Their conclusion was that methyl-ascorbigen acted as an endogenous formaldehyde generator. Enzymatic processes can continuously yield formaldehyde, and the generated formaldehyde had its effect in situ. At the same time, the otherwise slightly effective dimedone could capture the formaldehyde also in situ.

## CHEMICAL METHYLATION BY FORMALDEHYDE

The simplest type of methylation occurs without any enzymatic catalysis. This procedure is called either prebiotic methylation or chemical methylation. Waddel *et al.* [99] described their observation that certain prebiotic compounds (such as ethanolamine and glycine) can be methylated by an excess of formaldehyde alone. Direct methylation by formaldehyde is mechanistically analogous to the *de novo* origin of methyl groups by the reduction of methylene tetrahydrofolate. Moreover, the modern theory of cellular methyl transfer from S-adenosylmethionine to amine nitrogen may also involve formaldehyde as an intermediate, and subsequent reductive methylation.

Histones are DNA-binding proteins with relatively small molecular size. Histones are of basic character, rich in lysine and arginin, capable for N-methylation. $H_3$ and $H_4$ Histones of K562 erythroleukemia cells were successfully methylated by L-[methyl-$^3$H]methionine, in the presence of cycloheximide [100]. The function of histone methylation, like the bulk histone acetylation, remained to be determined.

Rhodopsin is an abundant membrane protein in the outer segment of rod cells in retina. Reductively methylated,

tritiated rhodopsin was prepared to the determination of rhodopsin by radioimmunoassay [101]. The procedure included reaction with formaldehyde in the presence of borotritide having high radioactivity. The product was purified using gel filtration and affinity chromatography. The reduced, alkylated and purified product retained its immunoreactivity.

## AMINO ACID MODIFIED BY VIRTUAL FORMYLATION

Oxygenases are specific enzymes that catalyze a direct incorporation of oxygen into the compound of question. Thereby the proper substituent is formed, such as e.g. hydroxyl or carboxyl. Dioxygenase is a specific member of oxygenases as both oxygen atoms are directly incorporated into the substrate. It is the tryptophan, which is modulated by one molecule of oxygen under the effect of tryptophan 2,3-dioxygenase, forming N-formylkinurenine. N-formylkinurenine contains both atoms of the molecular oxygen. When isotopically labeled oxygen was used for this reaction, both oxygen atoms were located at the carbonyl group of N-formylkinuranine [102].

## METHYLATION OF AMINO ACIDS

Pietrzak *et al.* [103] reacted apolipoprotein (apo) B-100 with formaldehyde under reducing conditions to form the epsilon-N-methyl lysine residue. They made antibodies against the methylated apoB-100, which cross-reacted effectively with the ethylated apoB-100. This later one was the product of the reaction of apolipoprotein (apo) B-100 with acetaldehyde.

Certain publications reported on the occurrence of methylation on the effect of formaldehyde. Tyihak *et al.* [104,105] demonstrated the methylation of L-lysine by formaldehyde in two tentative steps as given in Fig. (**6**). N-Hydroxy-methyl-lysine was considered as an intermediate. N-methyl-L-lysine was formed when the (second) formaldehyde reduced N-hydroxy-methyl-lysine. The oxydo-reduction gave N-methyl-L-lysine and one mol of formic acid. Similar reaction was postulated to take place in the case of peptides and proteins, as it was modeled in the case

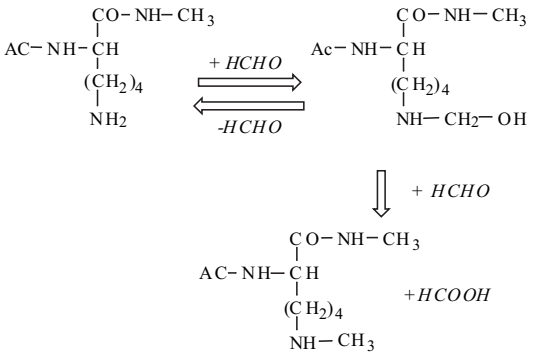


**Fig. (6).** Synthesis of $N^{\varepsilon}$-methyl-L-lysine was performed by the action of formaldehyde. Both the carboxyl group and the $\alpha$–amino group were protected [40].

of N-acetyl-L-lysine. After having the reaction with two formaldehyde molecules, the end product of the methylation was N-acetyl-N-methyl-L-lysine [104,105]. Identification was carried out using both thin-layer chromatography on Fixion 50X8 and NMR (nuclear magnetic resonance) spectroscopy [104]. The methylation reaction of formaldehyde was inhibited by the presence of ascorbic acid.

Monomethyllysine and dimethyllysine residues occur in certain muscle proteins and in cytochrome c. The calmoduline content of most organisms contain one trimethyllysine residue at a specific position. In some other proteins, Glu residues are undergone methylation, removing thereby their negative charge [106].

### Inborn error producing formaldehyde

Binzak *et al.* [107] described an inborn error of the enzyme system of the cascade from dimethylglycine through sarcopsine to glycine. Both enzymes [called dimethylglycine dehydrogenase (DMGDH) and sarcosine dehydrogenase (SDH) catalyze oxidative demethylation. Both DMGDH and SDH also utilize a non-covalently bound folate coenzyme that receives the "1-carbon" groups, which are removed by DMGDH and SDH, forming an "active formaldehyde". The inborn defect of DMGDH results in an unusual fish-like odor. Binzak *et al.* [107] have detected and located the polymorphism of hDMGDH cDNA sequence.

## INDIRECT EVIDENCES OF FORMALDEHYDE ACTION

There are several evidences, which indirectly indicate the presence of formaldehyde. Szende *et al.* [108] investigated the effect of (-)-deprenyl and (+)-deprenyl on the apoptosis of M-1 cell cultures. (-)-Deprenyl significantly decreased the apoptotic index (%), while the presence of (+)-deprenyl failed to influence it. The serum removal from A-2058 human melanoma cell culture caused apoptosis, which can be counterbalanced by the presence of $10^{-9}$ – $10^{-13}$ M of (-)-deprenyl. As the microsomal drug metabolizing enzyme inhibitor SKF-525A prevented from the anti-apoptotic effect of (-)-deprenyl, certain deprenyl metabolite(s) could be a candidate for this effect. A virtual contradiction was found that either one of the major (-)-deprenyl metabolites, such as nordeprenyl and methamphetamine, failed to induce a similar effect. However, a higher dose of (-)-deprenyl ($10^{-3}$ M) as well as the above mentioned deprenyl metabolites induced apoptosis.

Lajtha *et al.* [109] administered (-)-deprenyl and also p-fluoro-deprenyl directly into the rat brain striatum using microdialysis for 30 minutes. Samples were collected and the metabolites of (-)-deprenyl (and that of p-F-deprenyl) were measured. The method used conversion of the metabolites into their N-heptafluorobutyryl derivatives. Capillary gas chromatography was used for separation, with selective ion monitoring, negative chemical ionization with methane/ammonia reagent gas provided quantitation. *In situ* demethylation in the brain was proven by the appearance of both nordeprenyl and amphetamine, however, nothing was

mentioned about the determination of the generated formaldehyde. The conversion was rather high in the case of p-F-deprenyl. Lajtha *et al.* [109] consider that the cerebral metabolism of deprenyl is slow, and has no significant contribution to the pharmacological effect of deprenyl. However, both the generation and the level of formaldehyde, and the in situ brain effect of the generated formaldehyde remained neglected.

## Formaldehyde Generated by SSAO

Yu [110] described how SSAO (semicarbazide-sensitive amine oxidase) produces formaldehyde from methylamine. SSAO, located in the outer membrane of vascular smooth muscles and endothelia, catalyzes the deamination of methylamine. Formaldehyde and hydrogen peroxide is generated thereby, as depicted in Fig. (**7**) [15].

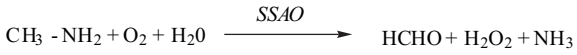


**Fig. (7).** Formaldehyde generation by semicarbazide-sensitive amine oxidase (SSAO).

The source of the endogenous methylamine can be epinephrine, creatinine, sarcosine, lecithin and nicotine [111,112]. Moreover, methylamine can be inhaled with cigarette smoke, and ingested from food and beverage [113]. After chronic administration of methylamine, the formaldehyde excretion in the rat urine definitely increased [15]. Also, elevated formaldehyde excretion was found in the case of streptozocin-treated diabetic rat [15]. It is remarkable that such type of deamination can be a source of the so-called aldehyde stress.

In addition, formaldehyde may cause the release of more SSAO, and thereby a cascade is produced, in addition to the inflammation and cytotoxicity. As an outcome of the increased presence of SSAO, vascular dementia and Alzheimer's disease can be induced [114-117].

Deng and Yu [15] demonstrated both *in vitro* and *in vivo*, that the SSAO mediated deamination of methylamine resulted in formaldehyde. There are several reaction routes resulting in formaldehyde, however, essential role is devoted to SSAO. Specific inhibitors of SSAO (such as MDL72974A [15]) definitely reduced formaldehyde formation, just as the formation of methylglyoxal and malondialdehyde was also reduced [15]. Yu *et al.* [114,115] have monitored other sources for aldehyde generation, such as formaldehyde and acrolein formation. Chronic administration of creatinine, a nutrition supplement augments the athletic performance, however, creatinine is converted to formaldehyde through methylamine. The elevated level of formaldehyde may cross-link proteins and DNA [114]. Semicarbazide-sensitive amine oxidase was described as the responsible enzyme converting allylamine (an industrial chemical) to acroleine.

The elevated level of SSAO is also involved in the pathogenesis of certain diseases, such as Alzheimer's disease and vascular dementia [116,117]. Formaldehyde may cause the release of more SSAO, and thereby a cascade is produced, in addition to the direct effect of formaldehyde on inflammation and cytotoxicity. As an outcome of the increased presence of SSAO, vascular dementia and Alzheimer's disease can be induced.

Meszaros *et al.* [118] suggested that an elevated SSAO level may cause endothelial injury. They connected the elevated level of generated cytotoxic metabolites (especially formaldehyde) as well as the increased oxidative stress to the initiation and progression of atherosclerosis and they concluded that effective and selective inhibitors of human SSAO might exert cytoprotective effects on endothelial cells.

## Assumption of Excited Formaldehyde

Tyihak *et al.* [63] postulated the formation of excited formaldehyde in plants among the stress conditions. Excited formaldehyde can be the reaction product between hydrogen peroxide and formaldehyde in the presence of lysine. Singlet oxygen is postulated as a byproduct of the reaction, which takes place under the conditions of either biotic or abiotic

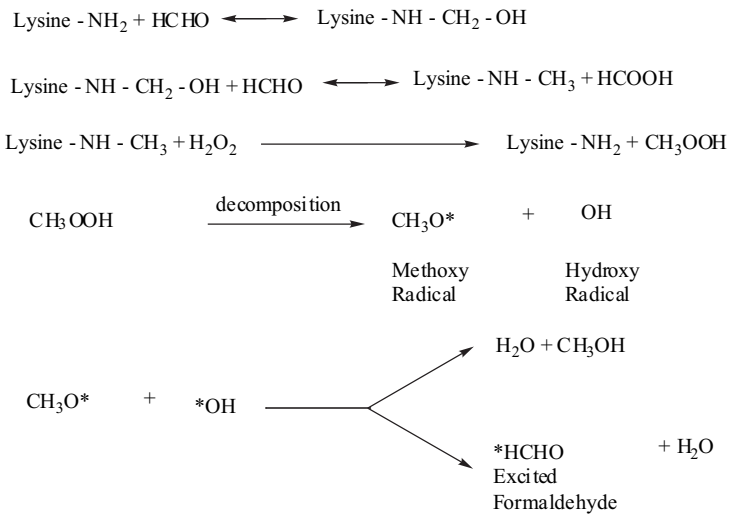


**Fig. (8).** The steps of generation of excited formaldehyde (*HCHO) [63].

stress. Extremely high reactivity is devoted to the reactive molecules, such as reactive formaldehyde.

Tyihak *et al.* [63] suggested a five-step reaction series to generate excited formaldehyde as given in the Fig. (**8**). They also claimed that the excited formaldehyde is extreme reactivity is a consequence of its richness in energies (3 eV that corresponds ~ 72 Kcal/mol) and of its short lifetime (25 msec).

## Where is Formaldehyde Generated in Humans?

The site of formaldehyde generation in the human body is not restricted to the liver where the first pass metabolism takes place. The importance of formaldehyde production in the brain cannot be overestimated. Formaldehyde is produced during oxidative N-demethylation of various endogenous and exogenous compounds *in situ*. Any compound present that has an N-methyl (O-methyl or S-methyl) group can be the potential source of formaldehyde.

## DNA Methylation

Certain nucleotide bases in DNA are sometimes enzymatically methylated. Methylation of both adenine and cytosine is more frequent than either of guanine or thymine. Methylation uses S-adenosylmethionine as a methyl group donor. In the case of E coli, methylation serves two distinct aims. It is related to the defense mechanisms of E coli, as its own DNA is methylated. It is distinguished from the foreign DNA, which is not methylated, and should be destroyed. The other methylation system of E coli methylates adenosine within the sequence of (5')GATC(3') to N6-methyladenosine. The latter is the Dam (DNA adenine methylation) and serves to repair mismatched base pairs formed through DNA replication [119].

About 5% of the cytidine residues in DNA are methylated in the case of eukaryotic cells. It is very common at CpG sequences, and is producing methyl-CpG units in both strands of the DNA. These cytidine methylations suppress the migration of DNA segments called transposons, and also have certain structural significance. The occurrence of 5-methylcytosine in an alternating CpG sequence increases the tendency for that segment of the DNA to assume the Z form [119].

Jensen *et al.* [120] reported methylation of DNA by microsomally activated dimethylnitrosamine. Hamster liver microsomal fraction and $^{14}C$-labelled dimethylnitrosamine were used. A good correlation was found between formaldehyde formation and DNA alkylation. The yield of radiolabelled formaldehyde was determined by precipitation in the form of formaldemethone. The methylated bases were 7-methylguanine, 3-methyladenine and 6-methylguanine.

Several reports have dealt with the hypothesis that drug alkylation of DNA via a redox pathway may be important in the cytotoxic processes [121-125]. Fenick *et al.* [121] used the formaldehyde conjugate of two antitumor drugs (doxorubicin and daunorubicin). The formaldehyde conjugates of doxorubicin and daunorubicin were called doxoform and daunoform, respectively. Doxoform is about 150-fold more toxic to MCF-7 human breast cancer cell, and 1000-fold more toxic to MCF-7/ADR resistant cell than the "parent" doxorubicin. Fenick *et al.* [121] interpreted the higher toxicity to resistant tumor cell with the higher lipophilicity of the derivatives.

Phillips *et al.* [122-124] reported that *in vitro* reductive activation of either doxorubicin or daunorubicin in the presence of DNA led to a transition blockade, possibly involving the alkylation and cross-linking of DNA by the reductively alkylated drug. Similar experiments were carried out by Skladanowski and Konopa, who cross-linked DNA by doxorubicin in HeLa S3 cells. They concluded that DNA cross-links induced cell apoptosis. The reductive activation of doxorubicin and daunorubicin plays a basic role in the production of superoxide and hydrogen peroxide [126-127]. Superoxide and peroxide, these two dioxygen species oxidize certain constituents of the medium to produce formaldehyde via the Fenton reaction [128]. The generated formaldehyde then connects between the 3'-amino group of the intercalated doxorubicin (or daunorubicin) to the 2-amino group of deoxyguanosine *via* a Schiff base. It is a virtual cross-link including one covalent bond from formaldehyde and one intercalative hydrogen bond with the opposite strand [121]. Cutts *et al.* [129,130] dealt with the interaction of anticancer drugs (anthracyclines) with formaldehyde. They desired the synergistic interaction of formaldehyde releasing a prodrug (pivaloyloxymethyl butyrate, AN-9) and adriamycine [130]. AN-9 is an anticancer drug candidate and adriamycine is a widely used anticancer drug. Their interaction was investigated in IMR-32 neuroblastoma and MCF breast adenocarcinoma cells. The relative timing of the addition of adriamycine and AN-9 was critical. When AN-9 was administered 2 hour after the cells were exposed to adriamycine, a twenty-fold enhancement of adriamycine-DNA adducts occurred. The enhanced level of these adducts and the accompanying decreased cells were directly related to the formaldehyde level, released by the esterase effect on AN-9. The relationship between the esterase-dependent release of formaldehyde from AN-9 and the decreased cell viability provides evidence for the formaldehyde-mediated activation of adriamycine.

Schlink *et al.* [131] studied the effect of environmental formaldehyde. Fifty-seven medical students were taking part in the observation of formaldehyde effects. Forty-one of them were exposed to 0.2 to 0.05 mg $m^{-3}$ formaldehyde during their anatomy course, 6 hour per week. Schlink *et al.* [131] did not find any difference between these medical students, and the control group. The formaldehyde exposure did not reduce the activity of the DNA repair protein O6-methylguanine DNA methyltransferase (MGMT). However, an earlier study on medical students in Cincinnati partially contradicts the results and statements of Schlink *et al.* [131]. In the case of the students in Cincinnati, the exposure to formaldehyde definitely reduced the MGMTR activity, increasing the cancer risk. However, the Cincinnati exposure was taking place at 2 mg $m^{-3}$ of formaldehyde, while the German students were exposed to one fourth to one twentieth of the formaldehyde level in the air.

Shank [86] carried out experiments to support his theory for the participation of formaldehyde in the toxicity of hydrazine ($NH_2$-$NH_2$). As it is well known, hydrazine is acutely toxic to the liver, kidney, and central nervous system. Hydrazine may react with endogenous formaldehyde forming a stable intermediate, tetraformyltrisazine. This intermediate is metabolized to an active DNA methylating agent. Both alcohol dehydrogenase and aldehyde dehydrogenase act as metabolizing enzymes. The methylated products, 7-methylguanine and O6-methylguanine were analyzed by HPLC and fluorescence spectrophotometry after the administration of 20 mg $kg^{-1}$ hydrazine to mice.

Mao [132] used piezoelectric quartz crystal impedance (PQCI) analysis for real-time monitoring the formaldehyde-induced DNA-lysozyme cross-link formation. PQCI provided multi-dimensional information. The observed frequency decrease ascribed to the mass increase resulted from the cross-linking. The kinetics of the cross-linking could also be quantitatively characterized by monitoring the frequency decrease.

Kato *et al.* [23] used mass spectrometric measurement of formaldehyde generated in human breast cancer cells treated with anthracycline antitumor drugs and also with formaldehyde conjugates of antitumor drugs. Four types of experimental series were compared: cells without any treatment (control), cells treated with the antitumor drug doxorubicin (DOX), and daunorubicin (DAU) and the daunorubicin-formaldehyde conjugate daunoform (DAUF). The lysate of untreated cells and drug resistant (MCF-7/Adr cells) untreated cells showed only background levels of formaldehyde. On the other hand, the formaldehyde level of either DOX- or DAU-treated cells were higher than the background and the formaldehyde concentration depended on the drug concentration (0.5 – 50 μm $mL^{-1}$), the treatment time (3 – 24 h) and cell density ($0.3 \times 10^{-6}$ – $7 \times 10^{-6}$ cells $mL^{-1}$). The lower limit for excess formaldehyde was 0.3 mM in MCF-7 cells treated with 0.5 M DAU for 24 h. Lysate of DOX- and DAU-sensitive cells treated with 0.5 micromolar equivalent of the formaldehyde conjugate (DAUF) for 3 hours showed only background level of formaldehyde. The results have proven a mechanism for drug cytotoxicity, which involved drug interaction of the metabolic process leading to formaldehyde production directly followed by the utilization of formaldehyde to virtually cross-link DNA.

Another drug-formaldehyde conjugate was suggested by Parker *et al.* [133]. Mitoxantrone can be administered in various neoplasm; it can be activated by formaldehyde *in vitro* to form a DNA adduct specific for CpG and CpA sites in DNA. *In vitro* transcription studies of mitoxantron-reacted DNA gave a three-fold enhancement in transcriptional blockage. *In vitro* cross-linking assay has also shown a 2-3 fold methylation-enhanced mitoxanthrone adduct formation. Methylation of cytosine at a single potential drug binding site on a duplex oligonucleotide also enhances the adduct level by three-fold. As the adducts of the methylated sites showed the same stability as non-methylated sites, cytosine methylation increases the accessibility of mitoxantrone to DNA rather than the kinetic stabilization of the adduct.

DNA-methylation of tumor supressor genes is a feature of human cancer. The cyclin-dependent kinase inhibitor gene (p16/Ink4A) is often hypermethylated. The p14/ARF gene (located 20 kb upstream on chromosome 9p21) is also methylated in carcinomas. Magdinier and Wolffe [134] reported on their study on the mechanism of the chemical treatment with 5-aza-2'-deoxycytidine (2-aza-dC) and trichostatin A and the methylated genes. The combined action of 5-aza-dC and trichostatin A results in robust gene expression. Methyl-CpG binding proteins and histone deacetylases seem to cooperate *in vivo*, and repress the expression of tumor supressor genes hypermethylated in cancer.

N-Nitrosomethylamylamine (NMAA) is a known carcinogen on the esophageal tract. Huang *et al.* [135] found that 2-phenylethyl isothiocyanate (PEITC) and 6-phenylhexyl isothiocyanate (PHITC) inhibit both the metabolism of N-nitrosomethylamylamine and its DNA methylation in rats. When the esophagi and the liver slices of the treated rats were incubated with NMAA and 10 M PEITS, the formation of hydroxy-NMAAs, formaldehyde and pentaldehyde was inhibited. A single dose of 0.1 or 1 mmol PEITS/kg reduced the O6-methylguanine level by 44-51% in the esophagus but by only 7-22% in the liver. PHITC inhibited NMAA metabolism by the liver, and also the methylation of guanine in liver DNA, but had a little effect in the esophagus.

In the studies of DNA methylation by NMAA [135], 7- and O6-methylguanine were determined by HPLC with fluorimetric detector. Huang *et al.* [135] support the view that 2-phenylethyl isothiocyanate may be a useful chemopreventive agent against esophageal carcinogenesis in humans.

Many RNAs of the mammalian cells are methylated at the N-6 nitrogen of adenylate residue within the RNA chain [136]. In the case of adenovirus infection, the methyl group may play some role of protecting the portion of the primary transcript that is to be preserved.

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