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Study of the Neurological Effects of Low Level Methanol in Normal Subjects and Subjects with Susceptibility to Folate Deficiency

CALIFORNIA ENVIRONMENTAL PROTECTION AGENCY
AIR RESOURCES BOARD
Research Division

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ABSTRACT

Methanol powered vehicles are being introduced in the U.S. as a solution to air pollution. Very little is known about methanol as an air pollutant and about the effects of low level exposure on neurobehavioral functioning. Folic acid plays an important role in formate metabolism and may influence the concentrations of this toxic metablite of methanol. The objective of this study is to provide information about whether or not acute exposure to methanol at the current industrial threshold limit value of 200 parts per million (ppm) for four hours would have adverse effects on human neurobehavioral, neurophysiological, and visual performances. Twenty six healthy subjects (male: n=15, female: n= 11; age: 26-51 years) were exposed in a chamber for four hours to methanol vapor (200 ppm, 260 mg/m^3) or to water vapor. The subjects served as their own controls in a double-blind design. Another six subjects from two possible folate deficient groups were also examined. The following endpoints were tested before, during or/and after exposure to methanol and water vapors: blood and urine formate and methanol levels; auditory-event-related potentials; visual performance; and behavioral tests. In an analysis of the results in the normal population, no important effects due to methanol exposure were found for the neurobehavioral and visual tests. However, possible effects were observed when outcomes were adjusted for factors possibly contributing to between-subject variability. For example, methanol effects were observed on P-300 auditory evoked potential amplitudes when adjusted for several variables, but particularly smoking and alcohol consumption.

Exposure to methanol at 200 ppm during the study increased the concentration of methanol in serum and the amount of methanol excreted in urine. No statistically significant differences in serum formate concentration were found between the exposure and control conditions at any time point or for area under the curve. Additionally, most of the test results from the folate-susceptible subjects did not demonstrate any notable deviation from the normal population of the study. One susceptible individual, while in a folate deficient state, accumulated formate after exposure.

In summary, while methanol concentrations reflected the exposure condition, no important neurobehavioral differences were found in the normal and susceptible populations after exposure to 200 ppm methanol for four hours. Because the study was not designed to examine the influences of age, gender, folate status, smoking and alcohol consumption, other more powerful and appropriately designed studies will be required to clearly demostrate methanol effects relating to any of these factors.

ACKNOWLEDGEMENTS

Patricia Chuwers, D.N.S., M.P.H., R.N., contributed collaboratively in all aspects of this project, including the development of protocols, recruitment of subjects, performance of exposure sessions, administration of neurobehavioral and neurophysiologic tests, data verification and analysis. This experience has been the basis for her doctoral dissertation.

John Osterloh, M.D. codeveloped the proposal for funding and directed the data verification and analysis phases. Also, he coordinated and supervised staff and academic contributors during the later stages of the study, including administrative and fiscal operations. Dr. Osterloh's laboratory performed methanol and formate analyses.

Charles E. Becker, M.D. co-developed the proposal for funding and carried out the early planning stages of this study. Initial medical screening of the recruited subjects was performed by Dr. Becker. In addition, he developed applications to the Committee on Human Research. He also served as advisor and coordinator for the study.

Tom Kelly served as statistician and performed data analysis along with other study group members. His suggestions and discussions have been invaluble.

Alessandra D'Alessandro, M.D. was a visiting post-doctural fellow in Dr. Osterloh's laboratory and assisted in many aspects of the study, especially the kinetic and biological monitoring considerations.

Hosna Moggadedi performed all serum and urine assays for formate and methanol. She developed and validated the new procedure for methanol analysis.

Patricia Quinlan, MPH, CIH operated the exposure chamber and monitored methanol production.

Yuen So, M.D. assisted in the choice of neurophysiologic tests and the assessment of the validity of P-300 measurements.

Patricia Buley, R.N. performed phlebotomy and Jeff Woodsley assisted in initial calibration of the chamber.

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DISCLAIMER

The statements and conclusions in this report are those of the investigators at the University of California San Francisco and not necessarily those of the State Air Resources Board. The mention of commercial products, their sources or their use in connection with material reported herein, is not to be construed as actual or implied endorsement of such products.

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SUMMARY AND RECOMMENDATIONS

The findings of this study support the current threshold limit value for methanol. Our healthy population was able to metabolize excess methanol burdens resulting from exposure without producing excess formate, its toxic metabolite and this exposure did not produce demonstrable neurotoxic effects. Some variables accounting for a portion of between-subject variablility, such as alcohol, smoking and folate status, may confound the detection of potential small neurophysiologic or neurobehaviorial outcomes. Also, it is shown that formate measured in serum and urine cannot be used as an individual biological indicator of methanol exposure at the TLV.

In future research, potential exposure scenarios should include a wider range of exposures to methanol including combinations with other chemicals, such as formaldehyde, alcohols and gasoline. Research should also be conducted in those geographical areas where there is folate deficiency or other special populations. For instance, in Brazil, where the population has been exposed in the last 4 years to a mixture of 60% ethanol, 33% methanol and 7% gasoline (Böhm & Saldiva, 1994). In addition, estimated risks to the fetus that have been based on rodent studies (Bolon et al. 1994; Nelson, 1985; Rogers, 1994) and should be tested with epidemiological studies.

INTRODUCTION

Methanol may become a major automotive fuel in the future (Health Effects Institute, 1987). Although gasoline fuel in California is unleaded, and diesel fuel has been reduced in sulfur and aromatics, these still contribute significant amounts of hydrocarbons, carbon monoxide and nitrogen oxides, which, together with sunlight, produce the high levels of ozone and other photochemical pollutants. Because emission control efficiencies for gasoline engines may be approaching their limit, a strong drive has been initiated to search for new or modified engines and fuels that are inherently cleaner. Financial incentives and public health mandates for the use of cleaner fuels have been formulated, and legislation has been proposed that would force the sale of alternative-fueled cars in polluted areas of the United States by 1995. The Clean Air Act Amendments of 1990 mandate that those areas that do not maintain the national standards for carbon monoxide, must add oxygenates, such as methanol, ethanol, methyl-tertiary-butyl ether (MTBE), tertiary-amyl-methyl ether (TAME), or ethyl-tertiary-butyl ether (ETBE), to their fuels. In Southern California, regional officials have endorsed a plan that would convert 40% of the cars to cleaner fuels by 1998. If enacted, the plan may ban the use of conventional gasolines in Southern California by the year 2007 (Gabele, 1990).

Methanol is considered to be both an economical and environmentally attractive alternative fuel, since methanol-powered engines can possibly operate with increased efficiency and lowered emissions, compared to a conventionally-powered engine (Gabele, 1990). Yet, a dramatic increase in methanol use as an automotive fuel would increase public exposure to this chemical and to formaldehyde which is a byproduct of incomplete methanol combustion. It is important, therefore, to evaluate the potential public health impact of such exposure. To predict occupational, dermal, and ingestion exposures to methanol, a wide range of exposures and conditions must be considered. In addition to methanol vapor exposure in the atmosphere, there will be increased exposure through skin absorption or through ingestion (either accidentally, or intentionally) during production, transportation, and tank filling. The water supply may become another route of ingestion if polluted by spills and leaks of methanol.

The United States Environmental Protection Agency (EPA) has predicted that the highest vapor exposure levels (< 500 ppm) to methanol will occur in residential garages, where cars are not optimized for methanol-fueled engines or malfunctioning; at self-service pumps during refueling; and in parking garages (150 ppm). By comparison, exposure concentrations in traffic, even with 100% usage of methanol-fueled vehicles, is thought to be very low (< 50 ppm). The exposure to methanol vapors in the worst case scenario would produce a methanol body burden of less than 1 mg/kg (Kavet & Nauss, 1990).

Methanol toxicity has been examined primarily at high doses after experimental intoxication in animals or accidental ingestions or exposures in humans. Clearly, methanol produces ocular and neurotoxicity at high doses. The nervous system is the critical target to assess in methanol toxicity, but little is known with respect to which specific neural processes are affected or which should be examined. In addition, susceptibility factors and the extent of human biologic variation in response to methanol are unknown. One such factor examined in animals is that of the effect folate-dependent pathways have on the metabolism of the putatively toxic metabolite, formate.

The main questions and objectives of this study are: 1) Whether there are neurobehavioral, neurophysiological, and visual effects at low levels of acute methanol vapor exposure (at the threshold limit value) in normal populations and whether individuals susceptible to folate deficiency are affected differently than normal individuals at the same level of exposure. 2) Whether neurobehavioral, neurophysiological, and visual outcomes are associated with the internal levels of methanol and formate in blood and urine, and/or associated with factors contributing to human biologic variation, such as gender, age, folate levels, alcohol consumption and smoking. 3) Whether formate concentrations are a useful biological monitor of methanol exposure. 4) Description of methanol absorption and elimination kinetics at threshold limit value (TLV) exposure doses. 5) This study will rectify shortcomings evident in previous studies of low dose methanol exposure including: lack of a control exposure; maintenance of a constant or known exposure of significant time; serial determinations of blood and urine concentrations of methanol and formate; dietary safeguards; measurement of pertinent toxic endpoints; randomization; and blinding. 6) In addition, the relevant literature on neurobehavioral effects and biological monitoring will be reviewed.

METHODS

General Design

In order to investigate possible subtle visual and neurobehavioral effects of methanol in humans, a randomized, double blinded, experimental exposure was conducted. Subjects in groups of three were exposed once to 200 ppm of methanol and once to water vapor (sham) for four hours, each subject serving as his/her own control. The exposure conditions were randomized and blinded to both the subjects and the administrator of the neurobehavioral tests. Only the industrial hygienist in charge of the methanol delivery system knew whether the vapor in the chamber was methanol or water.

Two separate groups were studied: normal men and women (n=26) and a population susceptible to folate deficiency(folate susceptibles,n=6). The folate-susceptible subjects participated in

the same procedure, but in groups of two. Four subjects with inflammatory bowel disease (IBD) were exposed twice, once to methanol and once to sham. Two psoriasis patients on methotrexate (MTX) were exposed twice to methanol: a first session during the peak effect of the patient's MTX therapy (folate-deficient status) and a second exposure in which the folate deficiency was corrected by folic acid supplements and the effect of the medication was minimal (one week a part of MTX intake).

Prior to the exposure sessions, training and adaptive experience with the seven neurobehavioral tests (NBT) were performed. Pre- and post-exposure testing was performed for each condition (exposed, sham). The main outcome was the change in NBT scores (post-pre) compared between the exposed and control exposure conditions. During each exposure condition, blood specimens were obtained at 11 time points during the exposure and 4 time points during four hours after the exposure. Just prior to each exposure session, at the end of the 4 hour exposure session and again 4 hours after the end of the exposure session, urine specimens were collected.

Sample Selection and Recruitment

The normal population was to be comprised of a convenience sample of males and females ranging in age from 21-51 years, multiracial, in good health, English speaking, and having normal hearing and normal or corrected vision. The exclusion criteria were a positive history of hepatitis or liver disease (methanol is metabolized in the liver), central nervous system disease (CNS disorders may influence outcome measures in NBT), color blindness or poor vision (limitation in the color discriminating test performance), sensitivity of having blood drawn, pregnancy, sensitivity to ethanol, and/or substance abuse.

The study protocol was reviewed and approved by the Committee on Human Research at the University of California San Francisco (UCSF). Advertisement flyers were posted at San Francisco General Hospital (SFGH) and the UCSF Health Sciences campus to recruit 32 normal subjects (See Appendix B). They were asked to contact the investigator's assistant, who then met or talked by phone with the applicants to discuss the procedure. An accurate description of the study goals, procedures, risks, and benefits of participation was given either by phone or in person at the time of recruitment. Those interested in participating were asked a series of questions to determine whether or not they were eligible. Of 36 subjects answering the advertisement, 33 met the inclusion criteria. From the 33 subjects in the normal population, one was excluded after the questionnaire and physical examination, because of a recent history of hepatitis B. Five of the 32 subjects recruited participated in a pilot study. All subjects were reimbursed $500 for their time (over 20 hours of participation) and inconvenience.

Those who met the inclusion/exclusion criteria were invited to an initial meeting where the following were performed: complete explanation of the study and acquisition of informed consent;

administration of a short questionnaire covering brief occupational and medical histories, including caffeine, alcohol and smoking consumption (see Appendix G); a brief physical exam; and initial training with the neurobehavioral and neurophysiologic tests.

Recruitment of the Folate-Susceptible Subjects

The sample size of the folate-susceptible population was determined by the limited availability and positive response of subjects. The susceptible population signed a consent form similar to the normal population but with slight variations. Initially, a folate-deficient population was sought. In the effort to recruit folate-deficient participants to the study, a number of approaches were taken. 1) The alcoholic and drug abuse center at SFGH was asked to collaborate, but the recruitment of subjects from their clinic was difficult administratively and potential subjects would likely be folate replete during the detoxification period. Also, because baseline neurologic status was often poor or changing and because these subjects were likely to have higher endogenous methanol concentrations, these potential subjects were not good candidates for the study. 2) An advertisement for the study was published in a newsletter for patients with inflammatory bowel disease without response. 3) In collaboration with a physician directing a gastrointestinal clinic at Kaiser Permanente, three patients were assessed initially and only one met the inclusion criteria, but the subject changed her mind before beginning the first experimental session. 4) Patients enrolled at the Inflammatory Bowel Disease (IBD) Clinic at California Pacific Medical Center were made available for screening through collaboration with that institution and approval of their Internal Review Board . A preliminary screening of over 400 patients at the IBD Clinic was conducted to detect the prevalence and incidence of folate deficiency, and the number of clients on medication or nutritional supplements. Forty potential subjects were identified. However, folate deficiency was not detected among any of the patients. Five patients were assessed as being very susceptible to folate deficiency due to their early enrollment at the clinic and history of disease. Four completed the exposure study. One was not accepted in the study because a long history of drug abuse. 5) Psoriasis patients on MTX were accessed through the Dermatology Clinic at UCSF. There was a client base of fifty such patients. After obtaining permission from the directing physician, the procedure was discussed with the patients. Two of the three patients that met the inclusion criteria gave consent and one of them was folate deficient (red cell folate < 160 ng/ml). Therefore, the susceptible group included four men and two women aged 21-64 years in otherwise good general health.

Because we originally proposed to have a population of 20 folate deficient subjects, but encountered great difficulty in locating such subjects, we inquired as to the documentation of our premise that such a population could be located. We had based our general estimates of the prevalence of folate deficiency on medical texts stating its prevalence to be 10% in the general population and as

high as 50% in populations with the diseases surveyed above (bowel disease, alcoholics, AIDS, poor diet, hematologic disease). It was clear we were not encountering such rates. Therefore, three limited surveys were performed. In over 700 blood tests ordered for red cell folate concentrations at San Francisco General Hospital during a four month period, less than 3% were low. Considering that the tests were ordered in a sick hospital patient population with some clinical suspicion of possibly having folate deficiency, this suggests that rates in other populations would be even lower. A similar survey of about 300 tests at UCSF showed a rate of about 1%. Also, in a developing database at UCSF covering 1986-1988 (2 1/2 years), only 88 abnormal red cell folate tests were discovered from inpatient and outpatient records (denominator unknown, but total admissions approximate 70,000).

The over-estimation of folate deficiency by us in our proposal was based on probable over-estimation in the literature due to the following. Older estimates (prior to 15-20 years ago) requoted in the literature, were based on testing done with a serum folate assay, which was microbiological in principal, requiring folate from the specimen to allow bacterial growth. This assay not only had poor sensitivity and specificity, but measured circulating forms of serum folate, which are not reflective of tissue folate stores necessary for folate-dependent pathways. Later and more precise serum folate assays also were subject to this problem. As tissue stores are depleted, serum folate will decrease sooner than tissue stores are depleted and before the individual manifests symptoms of folate deficiency. In addition, folate deficiency had become, unfortunately, defined by the test result rather than the clinical condition. As such, 95% reference intervals from normal populations defined normality, with the lower 2.5% being assigned as abnormal. These factors contribute to the overestimations of the condition. Red cell folate determinations have been thought to reflect tissue folate levels better than serum folate, though surveys of the prevalence of low red cell folate were not distinguished by us. However, Jaffe and Schilling (1991) found that serum and red cell folate levels were highly correlated but that a low red cell folate matched with the clinical outcome in only 5% of the cases. They concluded that the sensitivity and specificity of a low red cell or serum folate level in the diagnosis of ill or healthy individuals was undefined and that, until prospective studies utilizing newer, more specific biochemical indicators of tissue folate were completed, the interpretation of low folate levels would remain problematic.

Exposure Chamber and Research Setting

Controlled exposure took place in a 8 x 8 x 7.9 ft stainless steel exposure chamber located at the UCSF Cardiovascular Research Institute. The chamber had a total volume of 506 ft^3 and a ventilation rate of 11.86 air changes per hour. Air entering the chamber was filtered through a charcoal filter. The temperature was maintained at 20^{o} C and relative humidity was controlled at 40%.The

chamber was equipped with comfortable chairs, where the subjects were able to drink, eat, read, rest, talk and even play games with other participants. The chamber had a large window that permitted constant communication with the industrial hygienist.

A high performance liquid chromatography pump delivered either highly purified methanol or water from a small reservoir onto a hot plate (70°C), which vaporized the liquid. This vapor was circulated through the chamber by an oscillating fan located behind the plate (see Figure 1). Air samples collected at various zones inside the chamber (corners, ceiling, floor) and at the subject's breathing zone showed that methanol vapors were uniformly distributed. A Miran (Foxboro Analytical) infrared spectrophotometer placed in the chamber measured methanol concentrations at a wavelength of 9.53 μm. The instrument measurements as well as chamber samples were initially validated against gas chromatography meaurements (see Appendix M) and several times over the course of the study. Readings of 200 ppm were obtained when the true concentration was 201.9 ppm. The pump delivery rate was controlled by a personal computer, which received feedback of methanol concentrations from the spectrophotometer every 30 seconds. The methanol air concentration was stable within the desired range of 200 ppm ± 20 ppm (10%) (see Figure 2).

Neurobehavioral, Neurophysiological and Visual Tests

Neurobehavioral function was studied by means of a series of tests recommended by Letz et al. (1986, 1988, 1990, 1991, 1993) and Cook et al. (1991), which had also proven sensitive in other studies on solvents in the detection of early functional impairment due to toxic exposure. These tests were: 2 & 7, Sternberg, Stroop, and Symbol Digit. Event-related neurophysiological potentials (P-300) that correlated with cognitive functions and a wide range of neurological disorders were also recorded in this study. A battery of visual tests: Lanthony 15 Hue (a color-discrimination tests), and Vistech (a contrast sensitivity test) assessed the visual system of the subjects to determine whether methanol affected the visual system, and to determine what functions, if any, were affected.

Three of the researchers instructed the subjects on how to perform the various NBT tests, using a standard sheet for three of the NBT (see Appendix I). The Sternberg has its own computerized instructions. All the neurobehavioral training tests were given by one researcher. Subjects were trained in the first meeting before the experimental session on each of the tests to reduce the impact of learning effects and any anxiety related to test performance. In addition, administration of tests and exposure sessions were begun at the same hour of the day and, for women, at the same stage of their menstrual cycle. These standardizations were done in effort to reduce within-subject variation. The between-subject factors were reduced by having each subject serve as their own control.

Description and Review of NBT and Other Tests in this Study

In describing each test, its sensitivity (how well the test discriminates between exposed and nonexposed subjects), specificity (how well the test discriminates between the effects of different toxic substances), and reliability (test-retest results) are presented if they were available in the literature. Several studies on solvents, anesthetic substances, and anticonvulsant drugs describe the sensitivity, specificity, and reliability of the tests used in this study (see Appendix A).

2 & 7 Visual scanning performance has been shown to be a sensitive indicator of some drug responses, and the 2 & 7 test is similar to the visual scanning technique used by Trimble and Thompson (1983). The test is hypothesized to measure cerebral dysfunction in frontal lobes. The subject must select target stimuli (the numbers 2 and 7) from an array of visually-presented distractors. These distractors either are letters or numbers, and every 15 seconds the subject must continually switch from one set of distractors to another. The total test period is 5 minutes, and the dependent measure of the task is the total number of targets identified and the total number of errors (of omission or commission) (see Appendix J). The scores analysed in this study are the total number of targets identified. There are 135 responses per test period, which are hand scored for tabulation. The test begins with a 30 second practice trial during which questions may be asked or performance can be corrected by the investigator. The test-retest reliability coefficient of the 2 & 7 is not specified in the literature.

Stroop test Stroop (1935) suggested that the difference in color naming and word reading (of a color name) was due to colors being associated with a variety of behavioral responses (controlled process), while words were associated with only one behavioral response, reading (automatic process). It was the interference between the two that Stroop observed and was the focus of his study. In order to further study this relationship between color naming and word reading, Stroop devised the test which is now referred to as the Stroop Color and Word Test. Since Stroop's original work, the test has attracted considerable attention because of its high reliability (Jensen & Rohwer, 1966; MacLeod, 1992). Golden (1976) examined the test-retest reliability of the Stroop test in a sample of 30 subjects and reported reliability coefficients of 0.86 for the Word score, 0.82 for the Color score, and 0.73 for the Color-Word score. Franzen, Tishelman, Sharp, & Friedman (1987), in a sample of 60 subjects, found a reliability coefficients of 0.83 for Word score, 0.74 for the Color score, and 0.67 for the Color Word score. The test can be given in a relatively short time, requires only an elementary education, is not culturally biased, and can easily be translated into foreign languages.

In general, impairment in word naming is related to left-hemisphere brain injuries, while color naming is related to right-hemisphere injuries (this deficit is an inability to recognize and classify color hues) or left-hemisphere injuries (this deficit is an inability to attach a name to the color hue) (Golden, 1978). Hooisma,

Twisk, Platälla, Muijser, and Kulig (1988) found that a Color Word Vigilance task similar to the Stroop was sensitive to the effects of 0.03% blood ethanol, while other, simpler tests were less sensitive.

The version of the Stroop used in this study consists of three parts, each of which was presented on a separate sheet of paper. In the first part, the subjects were presented with columns printed with the words "red", "blue", and "green", and the subjects asked to read aloud the color names as quickly as possible. The second part consisted of columns of Xs printed in red, blue, or green ink, and the subjects asked to name the colors of ink as quickly as possible. In the third part, the subjects were presented with the words red, blue, or green printed in a contradictory color, and asked to ignore the word and name instead, the color of the ink. In each case, the score was the number of correct responses in a 45-second period. The results of the test can be presented in the formula : CW - {[COL * WRD] / COL + WRD} (each term represents the the number of correct responses when a color is presented as a differently labeled color name (CW), as a color with the properly labeled color name (WRD), as a color presented without a label name (COL)) or as Color word score separately.

Symbol Digit The Symbol Digit substitution test has been included in six major neurobehavioral test batteries as a measure of coding skills, attention, concentration, and information processing, and psychomotor slowing (Agnew, Schwartz, Bolla, Ford, & Bleeker, 1991; Gullion and Eckerman, 1986; Lezak, 1983).

Exposure to the organic solvent, styrene, showed a significant effect on Symbol Digit performance (Letz, Mahoney, Hershman, Woskie, & Smith, 1990). In a study investigating the neurobehavioral effects of chronic occupational exposure to mixed organic solvents (included methanol) among Japanese industrial painters, performance on the Digit Symbol test (the classic test from the Wechsler Adult Intelligence Scale) in exposed subjects was significantly lower than those in the control (Kishi, Harabuchi, Katakura, Ikeda, & Miyake, 1993). Letz (1990) cited Echeverria who conducted a dissertational study on ethanol. In 42 subjects, a performance decrement was found in Symbol Digit at blood alcohol levels of 0.06%.

The paper and pencil form of the test displays a key with symbols and their respective matching digits from 1-10. Below are blank rows with the symbols above. The subject must copy the appropriate matching digit for each symbol in scrambled order based on a key at the top of the page (see Appendix K). The score is the number of correct digits drawn within 90 seconds.

The test-retest reliability coefficients of the Symbol Digit approached the 0.90 level in a study by Arcia and Otto (1992), who suggested that it may be a useful complement to other instruments in clinical diagnosis or screening. Baker et al. (1985) measured the reproducibility of the Symbol Digit test in computerized form, and a correlation of the second day with the first was 0.92 and after a

month was 0.84.

Sternberg test Memory often is affected by exposure to environmental toxicants, and measures of memory are included in many neurobehavioral task batteries (e.g., NES, Williamson's battery). The Sternberg memory task (memory-scanning test) was selected for use in this study because it measures the speed with which the memory store can be searched, independent of decision time and motor response time (Sternberg, 1975). The Sternberg has been shown to be sensitive, but not specific, to mercury exposure, inorganic lead, and underwater environments (Williamson, 1990). The test-retest reliability coefficent of the Sternberg is not specified in the literature, but in the NES2 Users Manual the test is recommended for repeated measurement design (Letz & Baker, 1988).

In the Sternberg, the subject was shown a series of digits (2, 3, 4, or 5 digits) and then must indicate whether the test digit comes from a previously presented set (the "yes" button is pressed if the digit is present and the "no" button if the digit is not present). Responses are scored as correct or incorrect, and response latencies are recorded. The measurements recorded in this study were the mean reaction times for each set size (i. e., list length) and probe type (positive or negative). These times then used are to generate the two scores used for data analysis, slope and intercept. Little interaction occurs between the researcher and the subject, unless the subject requested assistance in remembering the task practiced in the first session. The test begins after a short practice trial to refamiliarize the subject.

Vistech (vision contrast test system) The contrast sensitivity vision test consists of three charts, each of which has five rows of sine-wave grating patches of different spatial frequencies or cycles per degree (cpd.): 1.5, 3, 6, 12, and 18. From left to right, each row contains, one sample patch and eight test patches. The contrast of the test patches ranges from zero contrast to contrasts above and below the visual threshold in steps of 0.1 log units steps. Contrast is the maximum luminance minus the minimum luminance divided by their sum. The gratings are tilted in one of three orientations: -15^{o}, 0, and $+15^{o}$. The contrast and orientation of the test patches are randomized for each row to control for guessing. The subject reads across the rows and indicates the orientation of the wavy bars in each patch: left, right, up, or blank. The last correct response for each row is plotted on a special evaluation form and the points are connected to form a curve (see Appendix L). The sources of light (close to a wide window during daytime) were measured by a light meter.

Supporters of the contrast sensitivity vision testing claim it can detect early signs of visual loss associated with glaucoma (Sponsel, DePaul & Kaufman, 1990), pituitary adenoma (Kurzer, 1986), vitamin A deficiency (Leguire, Pappa, Kachmer, Rogers, & Bremer, 1991), cataracts, and contaminated contact lenses (Ginsburg, 1987), and ocular toxicity associated with certain chemical agents, such as

solvents (Frenette et al., 1991). Frenette et al. (1991) found that lower contrast sensitivity scores in intermediate spatial frequencies were observed among former microelectronic workers, which possibly reflected neural alterations which may have resulted from exposure to the neurotoxic organic solvents used in many aspects of the work process.

This test may be useful in detecting methanol/formate effect because in a study by Ginsburg, Evans, McNinch, Blauvelt, & Siegal (1985), contrast sensitivity was measured for seven subjects who had different levels of blood alcohol content under phototopic (daytime) and mesopic (dusk) luminance conditions. In general, a blood alcohol content of less than 0.1 percent resulted in contrast sensitivity changes at all tested spacial frequencies.

Lanthony 15 (hue desaturated panel) The Lanthony 15 Hue desaturated panel is a color arrangement test which identifies mild-to-moderate acquired dyschromatopsia and trichromatic abnormalities (Lanthony, 1974; Lanthony & Dobois-Poulsen, 1973). It takes less than 5 minutes to complete and has been used in the past to identify acquired color vision loss (commonly blue-yellow) in solvent-exposed workers (Mergler & Blain, 1987). The test is administered by randomly placing 15 pastel colored caps before the subject, who in a darkened room, is required to place them in chromatic order. Standard illumination is provided by a 1150-lux fluorescent lamp positioned 30 cm above the caps. The subject has unlimited time to complete the task but must do it with one eye closed to evaluate the color vision of each eye. The test can be scored in a quantifiable format and has been shown to be sensitive to the effects of organic solvents (Blain & Mergler, 1986). In a study by Mergler, Blain , & Lagace (1987) among 23 workers of a paint manufacture plant exposed to solvent mixtures, they concluded that chromatic discrimination impairment, associated with solvent exposure, reflects neural, rather than ocular damage. For that reason this test is recommended in field batteries to evaluate neurotoxic effects of solvent exposure.

Neurophysiological Test(P-300) Routine laboratory studies, such as radiographical imaging of the brain (CT, MRI), and electroencephalography (EEG), generally do not reveal abnormalities in most occupational neurologic disorders. An event-related potential (ERP) refers to a large electrical potential that can be detected by EEG when subjects are required to discriminate different sensory stimuli. They can provide a means of investigating cerebral processing, because changes may appear in the ERP before conventional neurodiagnostic methods detect abnormalities (Goodin, 1978; Morris, So, Lee, Lash, & Becker, 1992). Different types of sensory stimuli can elicit an ERP, although auditory stimuli is the modality most commonly used. Auditory ERP also has been termed the P-300 potential, because it occurs with a latency of approximately 300 milliseconds.

Evoked potentials provide information about brain activity and sensory tracts from the site stimulated in the cortex. Neurotoxic lesions are more likely to be situated in the long sensory tracts

than in the cortex, and delays can be expected in the latencies of evoked potentials. Partial lesions in the tracts also can cause diminished amplitude and changes in the shape of the peaks of the evoked potentials (Seppäläinen, 1985).

A dose-dependent prolongation in P-300 latency has been detected in subjects acutely exposed to nitrous oxide (Estrin, Moore, Letz, & Wasch, 1988; Fowler, Pogue & Porlier, 1990) and in a study by Estrin et al. (1988), where subject were also exposed to nitrous oxide, a reduction in P-300 amplitude was detected. Persistent cognitive impairments also have been associated with acute exposure to hydrogen sulfide and abnormally prolonged P-300 latencies (Wasch, Estrin, Yip, Bowler, & Cone, 1989).

P-300 latency and amplitude were measured in this study. An IBM-PC compatible computer was used, and stimulus presentation and data acquisition were controlled by customized software. An amplifier bandpass of 1-30 Hz was used. The computer generated a series of tones at an intensity of 70 dB. Eighty five percent of the tones were at a frequency of 1000 Hz (frequent tone), and 15% at a frequency of 2000 Hz (rare tone). The frequent tone and the rare tone were intermixed randomly. Each subject kept a mental count of the total number of rare tones, and at the end of the two tests given in one session they were asked to report the number of rare tones counted. Scalp electrodes recorded the neural activity. Amplitude was measured from the P-300 peak perpendicular to the line from the preceding negative peak to the next negative peak, and latency was measured by the computer after the intersection of the ascending and descending slopes of the P-300 potential was marked off by the researcher (Lash et al., 1990).

Phlebotomy and Sample Processing

Blood samples were obtained by venous phlebotomy of antecubital veins. A registered phlebotomist performed all draws as part of the exposure team. The phlebotomist quickly entered the chamber to perform this duty. Monitoring of the air methanol concentration showed no deviation in air methanol concentrations (see Figure 2). Phlebotomy was performed at the following times: 0, 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, minutes and at hours 4, 5, 6, 7, and 8. Blood specimens were collected in serum separater Vacutainer tubes and centrifuged on site. Serum was decanted into a polypropylene tube (5 mL), labeled and immmediately refrigerated. Urine samples were collected by voiding directly into polypropylene containers (4 Liter) inside the exposure chamber. These were collected just before entering the chamber (random collection) and a 4 and 8 hours (timed collections). Containers were kept sealed between collections. At the end of the exposure/follow-up session, sera and urine were frozen at -70 C until analysis.

Formate Analysis in Serum and Urine

Serum samples in the study were analyzed for formate using a

modified enzymatic method with a colorimetric end point. Formate dehydrogenase was coupled with diaphorase and the reduced dye measured. The method has been demonstrated to be linear, specific, sensitive, precise, and accurate (Grady & Osterloh, 1986, see Appendix O for documentation). Sample volumes were increased (300 uL for urine and 60 uL for serum) to improve the assay sensitivity. The limit of detection was 0.5 mg/L, and the coefficient of variation was 10% at 12 mg/L. Samples with the same ID number were analyzed in the same batch, to reduce interassay variability in the two groups of exposure (methanol and sham). All analyses were performed in duplicate. Quality control consisted of reanalysis of initially analyzed samples in each batch run.

Methanol Analysis in Serum and Urine

Measurement of blood methanol were made by headspace-gas chromatography (Hewlett-Packard Model 5890) with a headspace autosampler (Tekmar 7000). A number of steps were taken to optimize this assay (see Appendix N for documentation of optimization and validity studies). Optimal sensitivity (0.5 mg/L) for 200 µl of serum (100 µl of internal standard) was achieved using a bath temperature of 95^{o}C, addition of 1 gm of NaCl and a sample equilibration time of 5 minutes. Intra-assay precision for serum methanol (conc. = 3.06 mg/L and n = 10) gave a SD of 0.287, and a CV of 9.4%; for aqueous methanol (conc. = 3.34 and n =18), the SD was 0.149 and a CV of 3.9%. Using these methods funded by this grant, the detection limit of the assay was improved five fold and the precision at least three fold over earlier assays. Actual paired precision was determined and calculated in serum from subjects run in duplicates. Quality controls and calibrators were aqueous and prepared in advance. Calibration was daily for each batch run and quality controls were assayed every ten injections (ie. 5 subject duplicate time points). Within run QC were to be within 2 SD of the pre-assay mean or the preceding samples were reanalyzed. Between run criteria were that the QCs for a particular run must have a mean value within 3 SE of the overall QC mean. This was to determine if any shifts occured between batch analyses. Also, duplicates were repeated if there was not a 15% agreement.

The folate level assessment in serum or red cells was performed in the SmithKline Beecham Clinical Laboratories, where the reference normal folate was scored as >160 ng/ml in red cell and > 3.1 ng/ml in serum. The folate levels were analyzed by radioimmunoassay for serum in the normals and RBC in the folate-susceptible group.

Subject Protocol

A summary of procedures in the subject protocol is presented in Table 1. Subjects were placed on a special diet for 24 hours prior to the study sessions to minimize their intake of aspartame or methanol (daily intake have been estimated to be approximately 3-11 mg/kg) and of medicines that could possibly raise endogenous formate or methanol concentrations. Endogenous methanol and formate may

arise from amino acid catabolism, demethylation of endogenous substances, methyl ester hydrolysis, and intestinal bacteria. Prior to the experimental sessions, the following foods were prohibited: alcoholic beverages, diet fruit and drinks that contain aspartame, fruit and fruit juices, vegetables, and roasted coffee. Ascorbic acid (Vitamin C) also was prohibited 3 days before the testing.

The first meeting was for 2 hours; the second and third meetings (exposure sessions) were 9 hours each. At the first meeting, explanations and details about the study were provided. The consent form included description about the study procedures, risk and discomfort, confidentiality, treatment and compensation for injury, benefits, and reimbursement. Questions were answered, the subject's bill of rights was read and the consent form signed (see Appendices C-H). A questionnaire was completed and a physical examination performed. In addition, neurophysiological, visual, and neurobehavioral tests were administered to control for learning effects and reduce stress in baseline performance during the first experimental session.

The questionnaire assessed demographic variables such as age, gender, ethnic background, and education. It also evaluated chemical exposure in hobbies or current or previous occupations, the use of protective equipment when working with chemicals, work shifts, medical history and habits (coffee and alcohol intake, cigarette smoking) (See Appendix G).

A physical examination assessed pulse and respiratory rate, blood pressure, weight, height, skin, eyes (including visual acuity), ears, nose, mouth, neck, lymph nodes, thorax, lungs, cardiovascular, and peripheral vascular and neurologic systems, abdomen, and mental status (see Appendix H). Blood was drawn to assess plasma folate values in the normal population or RBC in the susceptible population. The two experimental sessions were identical, the only difference being random methanol or sham vapor exposure.

During the first hour of the first and second session, the subjects completed the neurobehavioral, visual and neurophysiological tests; and an intravenous line was introduced. The next 4 hours involved chamber exposure, after which the subjects remained for additional 4 hours of testing. Subjects were divided randomly into groups of three, except that women and men were kept separate, for two reasons: To maintain approximately the same hormonal stage in the women by testing them 4 weeks apart between the two exposure sessions, and to maintain privacy when passing urine during the 4-hour exposure in a chamber without rest rooms.

Because the neurobehavioral battery took an hour to perform and the immediate effect of exposure was to be tested, some of the tests (2 & 7, Stroop, and Symbol Digit) were done by every subject in the last 30 minutes of exposure in the chamber. After exposure, the subjects finished the Lanthony, Vistech, P-300, and Sternberg tests.

At the end of the two-session exposure, the subjects were asked when the methanol vapor was presented to assess if the participant knew the session of methanol exposure in a higher rate than by chance.

Data Processing

The data processing of the NBT and other tests consisted of scoring, computer entry, verification, and storage of the data gathered. For the Sternberg, Lanthony, and Vistech scoring was done by computer; for the Symbol Digit, 2-7, and Stroop, responses were counted and scored on score sheets by the investigators; for the P-300, responses were interpreted by identifying the wave peaks from which a computer calculated the latencies, and the investigator measured the amplitudes. All data were scored and entered independently by two separate investigators at the end of the entire data collection. Errors of data entry were identified by comparing the two independent entries for each data file. All hand scored data were checked for errors a third time by comparing hand scores to computer entries.

All computerized data were stored in IBM-compatible floppy disks, using only the subject ID numbers, without any identification information. The originals files with the completed questionnaire, physical examination and test performances and scoring, were stored in investigator's office/lab.

Data Analysis

Statistical Analysis Systems (SAS) for Personal Computers software was used to analyze the data. The power of a t-test for matched pairs was calculated. For a sample of 26 subjects, a power of 0.80 and alpha of 0.05 enables an effect size of between one half to two thirds of the standard deviation (estimates of variability among the neurobehavioral test results) to be detected. For formate, a difference between exposed and control conditions of about 4 mg/L would be detectable and 2 mg/L for changes from pre-exposure baseline. For methanol, differences of 0.5 mg/L would be detectable between groups and for changes from baseline.

Initially, paired t-tests were utilized in the analyses of the neurobehavioral test outcomes: comparison between controls and exposed sessions prior to exposure; between controls and exposed sessions after exposure; between pre and post exposure to methanol; between pre and post exposure to sham; and the difference between the pre and post changes of the controls compared to exposed condition. When outcome variables were not normally distributed, non-parametric tests were used (Mann-Whitney U). Formate and methanol in serum and urine also were analyzed using paired t-test (control vs exposed). Subsequent analysis of the major outcomes (differences in the changes of the tests) was performed by a repeated measures MANOVA to account for multiple comparisons and interactive covariates.

Pearson correlation coefficients were computed for the following independent variables: methanol in serum and urine at hour 4; serum methanol area under the curve from zero to hours 4, 6, or 8 (surrogate independent variables); gender; age; order of exposure, folate levels; alcohol consumption; and smoking. The dependent variables in these correlations were the difference of the changes in neurobehavioral, visual, and neurophysiological tests (as described above). Independent variables were considered trendworthy if they showed correlations with a $p < 0.10$ (arbitrary screening criterion) and showed consistency for correlations among related variables. Selected trend worthy correlations were further examined by multiple regression analysis to see if other remaining covariates (gender, age, folate concentration, smoking, and alcohol consumption) affected the observed relationship. This process was undertaken to determine if between subject variability in these outcomes could be accounted for by a more precise marker of exposure (measures of methanol) or other covariates.

Alcohol patterns were described in terms of number of drinks per week from three different ranks and also were characterized as being higher, typical or lower than usual (see Appendix G). Smoking patterns were described in terms of number of packs per week from four different ranks (from 0 to >7) and also characterized as being higher, typical or lower than usual. Folate is entered as the serum folate concentration in ng/mL.

The susceptible population (n = 6) was examined by comparing their individual scores in the neurobehavioral tests with the score distribution in the normal study population. The formate and methanol concentrations in blood and urine also were compared.

Methanol Kinetics Analysis

While it was considered that such low methanol levels might not provide sufficiently precise data, a kinetic analysis has been attempted. Peak concentrations were identified at the time measured. Absorption and elimination profiles were observed and fits were attempted with common curve functions. Generally, either biphasic linear, linear or logarithmic functions with the highest correlation ccoefficient were accepted to describe the data. Underlying rate constants of absorption were determined by stripping the elimination rate constant from the observed absorption rate constant.

Estimated dose was based on air concentration, algorithms for minute ventilation rate (derived from body weight and breaths per minute, Adams, 1993) and a pulmonary retention fraction of 0.58 (Sedivec, 1981). The dose, rate constants and areas-under-the-curve (AUC) were computed in order to calculate total clearance rates and volumes of distribution. Formulas and abbreviations for computation are shown in Table 6. To demonstrate the total imprecision in the data, calculated volumes of distribution (based on estimated dose, AUC and elimination rate constants) were compared to known volume of distribution (based on weight). Renal clearances were calculated

from the serum AUC and the urinary amounts of methanol (see Table 6).

RESULTS

Pilot Study

A pilot exposure was performed on five normal subjects. This was done in order to standardize and gain experience with protocols, as well as to refine some procedures. From this it was learned that two-three subjects per exposure was the optimal upper limit on the number of subjects exposed in any one session, so as to keep pace with data recordation during NBT tests and specimen labeling during acquistion. Originally, it was proposed that electroretinograms (ERG) would be performed. Because these did not prove to be reproducible and because the ERG interfered with the other visual tests, they were discontinued. In addition, batch sizes for formate analysis and quality control materials were evaluated. Preliminary concentrations in serum samples allowed for adjustment of sample volumes to reagent volumes in the assays. Pilot exposures were not blinded and could not be used in subsequent data analysis. In addition, due to the low methanol concentrations encountered, a request to fund a new head-space gas chromatograph was made. All subsequent determinations were performed with this instrument, as validated above and Appendix M.

Exposure Sessions

In the exposure sessions of the normal study sample, methanol exposure was presented first 5 out of 9 times and water vapor presented first 4 out of 9 times. On two occasions, once for the normal sample and once for the susceptible sample, a decision to expose the subjects to methanol in the first session was changed because the spectrophotometer did not function correctly when the equipment was tested before the beginning of methanol exposure. To prevent missing a full day of testing, the investigator changed the exposure conditions from methanol to sham. The subjects remained blind, but the investigator was aware of the type of exposure.

At the end of the two exposure sessions, all subjects were asked whether or not methanol vapors had been present in the first or second experimental session. Subjects guessed correctly about the presence of methanol vapor 18 out of 26 times (not significant). The subjects did not indicate the detection of any odor in either of the exposure sessions. The primary investigator guessed correctly 100% of the time when methanol was present in the chamber. The reasons for this accuracy was the detection of a very slight methanol odor, understanding of the instruments, and the behavior of the industrial hygienist.

Data Integrity

For the P-300, subjects' results were excluded when the

investigator and the neurological consultant considered the wave form to be unacceptable. These decisions were blinded. Seven of the 26 subjects' P-300 results were deleted because of unacceptable wave forms in the post hoc analysis. Poor wave forms in any of the sessions would exclude all P-300 data for that subject (eg. three subjects had insufficient baseline P-300 wave forms). In the Symbol Digit test, one of the subject's results was omitted because of unreasonable score (a longer performance time may have been attributed incorrectly). In the other neurobehavioral and visual tests, missing or errored data were not detected.

In the data on the urine formate, one subject's urine results were excluded, because the large amount of ascorbic acid consumed interfered in the formate assay. In one of the susceptible subjects, the data on serum and formate urine is missing because a labeling mistake.

Over 3000 methanol analyses were performed (standards, QCs, and duplicates). Four subjects were excluded from the serum methanol interpretation, because the results obtained were not biologically plausible. This was part of a limited problem with the quality of the methanol concentration results. An analysis of all data points showed a number of unexpected concentrations (0-4 per subject out of 30 points per subject). Criteria used to judge points were of two types: Statistical outliers and improbable kinetic changes. Improbable kinetic changes included single concentration time points that were suddenly different than surrounding concentration time points. By removing values greater than the outer 5% and 95% bounds of the average rates of change observed in the absorption and elimination data, extreme points representing isolated changes were eliminated. Statistical outlier analysis based on averaging the surrounding points (n=4-6) and eliminating points greater than 3 standard deviations resulted in similiarly eliminated data. Ultimately, 5.6% of the 840 methanol data points (exposed and control data) were eliminated prior to interpretation or further analysis.

Investigation of the cause of the odd data points showed that the distribution was not random, but also not clearly associated with any other design or methodologic factors (order or date of exposure, order or date of analysis, exposure session, or gender). Quality control results revealed no precision or accuracy problems. Duplicates and repeated samples were always similiarly affected. Commercial sampling and storage tubes were assayed and proved no bias or contamination. Two speculative explanations were considered. 1) Microbiologic contamination has not been reported to cause aberrant methanol results, possibly because such very low (endogenous) levels are not routinely analyzed, but it is entirely plausible, in that, there are bacteria and fungi capable of producing methanol as has been reported for ethanol. Samples in this study were analyzed first for formate. In opening the sample container and allowing it to sit during analysis, partially covered by parafilm, such a contamination could be possible. There was a partial association between odd methanol levels and the batch of formate analysis, but this was not

always true. An extra peak was noticed in many of the chromatograms for methanol analysis, possibly methyl formate. Though this peak was found not to be associated with the odd methanol levels, the presence of this peak showed a weak correlation with formate batch analysis number. Methyl formate could also have been produced by a microbiological organism. 2) The various labeling pens used to write identification marks on the outside of the serum containers may contain methanol or methyl formate. It is possible, though considered less likely, that these solvents could penetrate into the container wall. Experiments with ink labeling failed to show such a contamination, though the ink itself (in large amounts) produced a peak at the retention of methyl formate.

Subject Description

The original subject number in the normal population was 27 (15 male and 12 female), but 26 completed the research study, because one subject was eliminated after the second session for sensitivity to having blood drawn. Table 2 summarizes the distribution of age, race, education, weight, height, serum folate, smoking, and alcohol consumption by gender. Ages ranged from 26 to 51 years. The mean age and standard deviation for all subjects was 35.7 ± 6.8, with a median of 35 years. The mean and standard deviation for the age of the females was 39.6 ± 6.9 and for the males, 33.0 ± 5.7.

Most (59.7%) of the subjects finished college or trade school, a minority (15.4%) finished only high school, and the rest (26.9%) of the subjects finished graduate school. Most of the subjects were white; the minority representation was 30.8%. The mean weight of the normal study population was 172 ± 34 lbs, and ranged from 113 to 250 lbs, with a median of 171 lbs. The mean weight of the males was 187 ± 32 lbs; for the females, it was 151 ± 27 lbs. The mean height of the males was 72.5 ± 2.4 inches, and 65.6 ± 3.6 inches for the females.

Nineteen out of 26 were nonsmokers; all of the females were nonsmokers. Alcohol consumption (including beer and wine) was classified into three categories: 7 glass equivalents or less per week; 8 to 28 glasses per week; more than 28 glasses per week. None of the subjects admitted to drinking more than 28 glasses/week. The mean serum folate level, which was taken only once at the first meeting, was 10.3 ± 4.9 ng/mL, and ranged from 3.8 to 25.2, with a median of 8.9. The mean and standard deviation of folate in females was 11.7 ± 6.4 ng/mL, and in males, 9.2 ± 3.3.

Methanol Air Concentrations

Concentrations of methanol in the air were measured every thirty seconds by the infrared spectrophotometer. Concentrations were demonstrated to be constant at 200±10 ppm. Graphs of concentrations for each session were examined. An example is shown in Figure 2.

Methanol and Formate in Serum and Urine

The means and standard deviations of formate and methanol in serum and urine from the two exposure sessions are summarized in Table 3. The results of only 4 (hour 0, hour 1, hour 4, and hour 8) of the 15 time points (between hour 0 and hour 8) have been presented to simplify the data discussion (all data points are used for calculation of area under the curve). Mean concentration time curves for all time points and subjects are shown in Figures 3 and 4. All three samples (hour 0, hour 4, and hour 8) of urine were analyzed for formate and methanol and are also presented in Table 3.

Formate concentration in serum and urine did not show any statistically significant differences between the two exposure conditions. For example, the mean serum formate concentration at hour 1 was 10.9 ± 5.4 mg/mL during methanol exposure, and 11.7 ± 6.5 mg/mL during sham exposure. At hour 4, the mean serum formate concentration was higher during methanol exposure than during sham exposure (14.3 ± 8.9 versus 12.7 ± 6.4 mg/mL), although this difference was not statistically significant ($p = 0.39$). The difference in urine formate concentration between the two groups at the end of exposure (hour 4), approached a statistically significant value ($p = .08$).

Methanol at 200 ppm for 4 hours of chamber exposure increased the concentration of methanol in serum and urine. Peak serum methanol concentrations at four hours were predicted to be a maximum of 6 mg/L if no elimination occured. The peak increase over control levels was a mean of 5.1 mg/L, comparing favorably to predictions. Serum methanol, when comparing the control exposure to methanol exposure, was statistically significantly different at most time points. Only at baseline (hour 0), the serum methanol in the exposed and control conditions did not differ ($p = 0.20$). Subjects during the control exposure maintained a stable level of serum methanol during the entire 8 hours of the study. Occasionally, control subjects showed a slight upward increase in endogenous methanol concentrations during the 4 hours post exposure. This was thought to reflect dietary contributions since the subjects were allowed to ambulate and eat more during that period. In the exposed condition, after 1 hour exposure to methanol, there was an approximately several fold increase in methanol serum levels when compared to the initial level and a more than four fold increase over initial levels by 4 hours. The decrease of serum methanol began after exposure ceased and did not reach initial levels within the four hour post exposure follow-up period.

The area under the curve after 4, 6, and 8 hours exposure for serum methanol (SMauc4, SMauc6, SMauc8) were greater for the exposed condition. The excess areas under the curve in the methanol exposure condition minus the area under the curve in sham exposure were (mean and SD): 14.2 ± 4.4, 21.7 ± 6.8, and 25.9 ± 10.1 mg*hour/L, respectively. The urine methanol concentrations for the two collection periods were significantly different between exposure and control sessions, nearly five fold higher at both the 4 and 8 hour collections.

Methanol Low Level Kinetic Analysis

Only 19 of the 26 subjects had sufficient data for a complete kinetic analysis. Four subjects were eliminated from consideration because of methanol levels thought to be due to contamination (see above). Three other subjects showed either very flat absorption or elimination profiles making the calculation of AUC and derived terms quite imprecise.

Absorption profiles showed an initial rapid uptake phase followed by a nearly zero order secondary phase. These were fitted with either a biphasic linear or logarithmic functions. Fits are shown in Table 4. Elimination kinetics were first order and described by their first order rate constants (Ke). The mean elimination half-life was 2.62 ± 1.17 hours. To approximate the underlying absorption rate constant (Ks), the apparent first order absorption rate constants (Ka) were stripped by subtracting the first order elimination rate constant (Ke). Kinetic data are shown in Table 5. Renal clearances were demonstrated to be a very small percentage of the total clearance, as would be expected from observations in overdose kinetics. Most subjects did not return to baseline or control methanol concentration values within the four hour follow-up period. The predicted time to return to baseline (mean methanol concentration during the control period) was 13.24 ± 6.37 hours after the beginning of exposure.

Age, gender, weight and folate concentrations were tested to examine these variables as covariates of the main kinetic outcomes: SMauc8, Ke, weight adjusted total clearance (Cl_{tw}) and the initial rise in methanol absorption (SMj). Only gender was significantly associated with Ke ($r=0.4946$, $p=0.0313$) and age was associate with Cl_{tw} ($r=0.5166$, $p=0.0236$). The SMj was nearly associated with gender ($p=0.0550$) as was weight ($p=0.1022$). Interrelationships within either exposed condition for related measures of methanol and formate were also observed and were expected. For instance, SM4, SM8 and SMauc8 were all strongly intercorrelated. Serum formate concentrations at various time points were occasionally, but weakly intercorrelated, as were urine formates, though urine and serum formates were not correlated.

To demonstrate the imprecision of this low-level kinetic data, predicted volumes of distribution (Vd_p) were regressed with estimated (Vd_{est}). The regression was not significant ($r=0.3568$, $p=0.1338$). The explanation for this was apparent from the plot of the distributions of the those variables. The Vd_{est} varied 8 fold versus only two fold in Vd_p. The cumulative errors and variability in the computation of Vd_{est} (from extrapolated AUC, Ke, air concentration, breaths per minute, and body surface area) clearly contributed to this imprecision of this term.

Neurobehavioral and Visual Tests

Tables 7 summarizes the results in the normal population and their comparison by t-test. Table 8 shows the differences and changes in the test scores. Baseline neurobehavioral test scores in the two sessions (pre-chamber) were compared to ensure that initial test circumstances were similar. No test demonstrated any difference between the two (control and exposed) pre exposure testing circumstances. The comparison of the test scores for control and exposed conditions at the end of the exposure sessions (post exposure) showed the test scores to be the same, except for the Vistech test at 1.5 cycles per degree ($p=0.0120$). When the paired changes in scores (post minus pre) over either of the exposure sessions are examined, a number of test scores showed improvement (for the control exposure: Vistech at 1.5 cpd; in both exposed and control exposures: Symbol Digit, Stroop Formula and Color word, Vistech at 18.0 cpd; in the exposed only: Vistech at 12.0 cpd). Improvements due to learning effects may be the explanation for this during either exposure condition. However, while there were these within-condition trends, the change in test scores during methanol exposure were not different from the change in scores during the control exposure, except for the Vistech at 1.5 cpd ($p=0.0385$) and nearly so for the Symbol Digit test ($p=0.0625$). For the Vistech contrast sensitivity test at 1.5 cpd, the changes in the scores over the two exposure conditions were in opposite directions. It is possible that putative learning trends in the control exposure condition were offset by lesser trends in the exposed condition for some tests. Also, the improvement in the Symbol Digit score during the control exposure ($p=0.0001$) may have been offset by the lesser change during the methanol exposed condition ($p= 0.0849$), negating a significant difference when the two trends were compared.

P-300 amplitude decreased during the exposure to methanol ($p = 0.0061$), but this change was not statistically significant when compared to the change during the control exposure. Where significant changes in test scores occur during one exposure, but are not different from changes in the other exposure condition, it must be concluded that these are either real changes due to methanol that are not statistically significant because of the inherent variability and sample size, or such changes are random chance.

It is important to note that the increased number of comparisons may increase the chance of randomly finding statistical significance. The major hypothesis of methanol exposure affecting any one test outcome is best represented by the comparison of the changes in the two exposure conditons, in that it entails the fewest comparisons and includes all the variability. Repeated measures-analysis of variance will take into account multiple comparisons and the within subject variability. Interactions of exposure condition and the covariates that may affect between subject differences can also be tested. Using MANOVA, difference in the changes in P-300 amplitudes significantly varied with exposure condition alone, with the interactions of exposure condition and alcohol use or exposure and smoking history. For P-300 latencies, only interactions of exposure

and gender or exposure and serum folate were significant. For the Symbol digit test, exposure alone or interactions of exposure and age together were significant. For Vistech 18 cpd, interactions of exposure and folate together were significant. Thus, an exposure effect is noted for P-300 amplitude and Symbol Digit considering within subject variability and when variables are considered that might account for between subject variability.

Because test outcomes could also be related to a more precise indicator of internal exposure (ie. meaures of serum methanol) as opposed to categorical or condition designation (exposed, control), additional statistical tests were performed to determine whether underlying relationships existed. Pearson correlations were performed using serum and urine methanol at hour 4 (SM4 & UM4); serum methanol area under the curve at hours 4, 6 and 8 (SMauc4, SMauc6, SMauc8) as markers of exposure extent. Also, folate levels, gender, age, alcohol consumption, and smoking were considered confounding or covariates variables. These variables were tested for their correlation with the difference (exposed-control) of the changes (post-pre) in the test scores. Serum formate in blood and urine were omitted in the correlations because their relatively large variability and because their concentrations did not change during or due to exposure. Correlations were selected as interesting if $p < 0.1$, and if a correlation was also shown for related tests or with several measures of methanol exposure (see Table 11). The following test outcomes of interest were chosen for further examination: P-300 amplitude and latency, Vistech 1.5 and 18 cpd, Symbol digit and Sternberg.

Multiple regression analysis was performed on these selected test outcomes or those that significantly changed after methanol exposure (see above). Predictor variables for the multiple regressions were serum methanol area under the curve at hour 4 (SMauc4 as being the most dynamic measure of methanol in the body prior to testing), gender, age, folate levels, alcohol , and smoking consumption. As stated above, the SMauc4 was entered as a more precise indicator of exposure within the exposed condition as methanol concentrations varied between individuals though external exposure was the same. Type I and Type III sum of squares (SS) were used. In Type I SS regression results are dependent on the order in which the independent variables are entered, each subsequently entered variable can explain only the remaining portion of the variability (SAS Institute, 1988). In Type III, each effect of each variable is adjusted for all included variables. The comparison of the two SS types are useful to detect redundancy and suppression by one covariate with the other.

For P-300 latency, Symbol Digit, Sternberg (intercept and slope), and Vistech 1.5 cpd outcomes (difference of the changes during exposures), the variability from all variables (mentioned above) together, including variability in exposure (SMauc4), did not significantly account for the variability of the differences in the changes in scores. However, the variability in the differences in

the changes in the P-300 amplitude was significantly accounted for by all these variables together (r^2=0.8356). SMauc4 varied inversely with difference in the change in P-300 amplitude (p=0.0060), when entered as the first variable, with smoking use (also inversely) and alcohol use significantly covarying (type I SS), but some interaction is present for SMauc4 with the other variables, since when all variables are adjusted to each others contribution, SMauc4 is no longer significant, where as smoking and alcohol remain. For the Vistech (contrast sensitivity) at 18 cpd, SMauc4, folate concentration and smoking inversely accounted for a smaller portion of the variance (r^2=0.6553), than in the P-300 situation. However, the SMauc4 remained significant (Type I and III), as did folate concentration and smoking use.

Susceptible Individuals

The test results of the folate-susceptible population are presented in Table 9 and Table 10. In four of the subjects (IBD patients), the results were compared to the reference range of the normal population for the difference in the changes during the two exposures for any test outcome, since these subjects were studied in the same design. The other two subjects (MTX patients) were compared to the reference ranges of the normal population for the change in test scores during the methanol exposure, because they were exposed twice to methanol.

Table 10 presents individual test results in the susceptible population by identification number. Subjects 802, 803, 903, 807 and 907 scored within two standard deviations from normal in all the tests. Subject 806 scored lower than two standard deviations from the normal mean in the Vistech 1.5 and 12 cpd (difference in the exposure changes) .

Methanol and formate in serum and urine in the susceptible population are presented in Table 9. Methanol levels were found to be similar to the normal population, but differences were noted in a few isolated formate levels. The interval excretion of formate from 0-4 hours by subject 806 was multiple standard deviations above the normal mean, but was normal for the 4-8 hour interval. Subject 907, while on MTX (and therefore blocked formate metabolism), was more than two standard deviations above the mean for serum formate at hour 1 and at hour 8 during methanol exposure and also for urinary formate excretion during the 0-4 hour interval, but not the 4-8 hour interval. When subject 907 was off MTX and exposed to methanol, urine and serum formate amounts were similar to the normal population.

DISCUSSION

The results of this study demonstrate that 4 hours of methanol exposure at the current threshold limit value does not affect formate

concentration in serum and formate excretion in urine; nor does this exposure affect neurobehavioral, neurophysiological, and visual performance in the normal population. However, there are some interesting findings in the results that should be discussed.

There were significant paired changes (by t-test) in some test scores over the periods of exposure, but these changes were not significant when compared to control changes in test scores. The Symbol Digit test scores changed during the control exposure, but not during the methanol exposure. This may be due to suppression of a learning effect by exposure to methanol. In the Stroop test, there was an improvement of test scores after 4 hours of exposure in the chamber (either sham or methanol), which also may be related to learning effect or increase in motivation and attention (administration of a test in the last half hour in the chamber was a sign that the exposure was about to end). Because of the many comparisons of change made with t-tests, the likelihood of random chance must be considered.

Though a simple analysis by t-test suggested that the contrast sensitivity at 1.5 cpd was affected by methanol exposure (comparison of the changes over the exposure period was significant), this appears to be randon chance since neither MANOVA (which will consider within subject variability) nor regression analysis (which could account for some between subject factors) yielded any further findings. However, it is possible that the change in Vistech 1.5 cpd scores during the control exposure and the non-significant change during methanol exposure may be associated with the effect of methanol on other variables (e.g., motivation, concentration), that could affect this specific visual test performance. This is considered unlikely.

The MANOVA analysis is the most important analysis with respect to the effect of exposure on the test outcomes because multiple comparisons and within subject variation are considered. Two of the outcomes showed an effect of exposure, the P-300 amplitude and the Symbol Digit, with possible interactions due to variables that could explain some between subject variability.

If methanol exposure actually does alter neurobehavioral performance and if test outcomes were related to other between subject factors such as internal dose (SMauc4), age, gender, folate levels, alcohol consumption and smoking, then multiple regression analysis may identify some of these influences. Using SMauc4 as a more precise marker of internal exposure to explain within exposure (between subject) variability, multiple regression analysis demonstrated that the Vistech at 18 cpd and P-300 amplitude were associated with internal methanol exposure when other variables accounted for some of the remaining variability. Though finding some similar relationships as in the MANOVA analysis, regressions are difficult to interpret since several of the regressions are negative and counter-intuitive. For instance how would higher levels of folate or higher alcohol intakes lead to improvements in P-300

amplitude during methanol exposure. While the effect of some of these between subject variables are not clear, further investigation may be warrranted. Because the number of subjects was reduced in each analysis due to data validity considerations, possible bias may also be operant in these regression findings.

In most of the neurobehavioral tests, results from folate-susceptible subjects did not demonstrate any particular deviation from the normal study population. The only test that may have shown some sensitivity was the Vistech contrast sensitivity test, where four of the six subjects scored more than two standard deviations below the mean in one or more of the Vistech sub-tests. Vistech may detect impaired visual performances more readily than Lanthony, the color discriminating test.

Three of the six folate-susceptible subjects had urine formate concentrations at hour 0 which were higher than two or three standard deviations above the mean of normal population. The reason for this possible increased production of formate in these three subjects is unknown. Alteration of bowel flora or metabolic clearance are speculative possibilities. When exposed to methanol, one of the susceptible subjects was folate deficient due to MTX therapy, and experienced an increase in formate levels. The level of formate was not reduced until 4 hours after exposure (hour 8). On a repeated second exposure, the red cell folate level had returned to normal, the same subject demonstrated lower levels of formate only at hour 8. This finding has not been demonstrated in the literature in humans and is compatible with the hypothesis that folate deficient populations may accumulate formate during methanol exposure. This single case strengthens the need for further investigation on the susceptibility of folate deficient population to methanol exposure.

After exposure to 200 ppm methanol for 4 hours, serum formate, which is a measure the proximate toxicant in methanol poisoning, did not change. Exposure to methanol at 200 ppm clearly increased the concentration of methanol in serum and the amount of methanol excreted in urine. Thus, methanol in serum and urine are good biological markers for low-level methanol exposure and formate concentrations are not (see review for related studies).

Because the reported half-life of methanol in blood and urine is approximately 2.5-3 hours (Sedivec et al., 1981), an exposure time to methanol vapor of 4 hours permitted limited pharmacokinetic assessment. It was predicted that large variability would exist in such data because of the impact of endogenous metabolism and dietary processes on the low measured concentrations. One way to confirm this, apart from the large standard deviations, was to compare the calculated volume of distributions to those predicted based on body weight. The calculated volumes of distribution includes the greatest number of sources of error for any of the pharmacokinetic terms. Therefore, all other terms would be more precise. Not only was the standard deviation six times as large for the calculated volumes of distribution, but these were biased as well. Therefore these kinetic

data at very low methanol concentrations should be considered very approximate. The major error components of calculated clearances and volume of distributions comes from the estimated dose and extrapolated AUC. Estimated dose is based on minute ventilation according to the formula of Adams, 1993 (based on breaths per minute and heart rate measured only once at rest and body area estimates from body weight), a fixed pulmonary retention value of 58%, and a fixed methanol concentration 250 mg/m^3. Obviously, three of the five terms can be quite variable. In fact, in the report by Adams, the correlation between measured minute ventilation and estimated gives correlation coefficient values of only around 0.5. To compute clearances, the AUC is divided into the dose. The AUC term includes the actual measured area-under-the-curve plus the extrapolated portion based on the measured rate elimination constant (Ke). Because of the variability in Ke, this added extrapolated AUC will be quite variable. While the calculated terms (volumes of distribution and clearance) have large compounded imprecision, the Ke is directly measured and converts to half-lives with a mean of 2.62 hours, which is consistent with half-lives measured at higher concentrations. Also, the negligible contribution from renal clearance is demonstrated.

Limitations

Although double blind testing was desired, the ability of the investigator to correctly identify exposure resulted in the lack of a full double blindedness. However, scoring was performed only at the end of the study and would have prevented possible observer bias.

In order to produce a particular behavior, a critical level of attention is required (Williamson, 1990). Attention must be measured separately and it may not be possible to determine if apparent deficits in memory, reaction time, or other performances were due simply to poorer attention, since the subject must fully attend to the task in order to perform it at an optimum level. However, when a large battery of tests are given, attention measured in one test, does not indicate the same level of attention for consecutive tests.

Although the fact that the subjects were their own control diminished many possible confounders, unknown variables may be at work. For example, stimulus presentation in Symbol Digit, Sternberg, Stroop, and 2 & 7 is visual in nature, and since visual functions may be affected by methanol, then, vision may be a confounder.

The sample size of 26 subjects may detect only an effect size of one-half to two-thirds of the standard deviation for any measure. In the case of low-level exposure to a chemical, a smaller effect size is preferable to detect subtle effects. The inherent and additive variability in NBT type tests is problematic, but this study design is the most powerful and specific approach. This study does remedy a number of deficiencies found in previous studies including: lack of a control exposure; maintenance of a constant or known exposure for a significant time; serial determinations of blood and urine

concentrations of methanol and formate; dietary safeguards; measurement of pertinent toxic endpoints; randomization; and blinding. Although exposure to a range of methanol vapor levels would be preferred, funding was provided for two levels. Zero (no exposure) and the current TLV were chosen as the most useful.

In the San Francisco Bay Area, recruitment of folate deficient subjects, even among the most susceptible populations, is problematic. Folate deficiency in hospital populations was far less than expected. Defining folate deficiency without clinical signs and symptoms is difficult based on folate analysis alone.

In the measurement of very low methanol concentrations, underlying contributions from diet and metabolism are considered to be significant. Concentrations at baseline and during control exposures for some subjects were as high as concentrations during methanol exposure for other subjects. Also, a few subjects showed slight post control exposure changes in concentrations, indicating that activity or diet may play a role in determining endogenous concentrations.

REVIEW OF THE METHANOL AND FOLATE LITERATURE

Methanol

Methanol, (methyl alcohol, wood alcohol) is a commonly employed organic solvent and reactant in organic synthetic procedures. It is used in a variety of consumer products, such as solid fuels, solvents in photocopy machine solutions, gas-line antifreeze, paint strippers and removers, model airplane fuels, racing car fuel, and windshield washing solutions (Constantini, 1993; Litovitz, 1990; Tephly, 1991). Derivative products of methanol include formaldehyde, methylamines, dimethylether, and formic and acetic acid (Calkins, 1984). Crude methanol has a disagreeable taste and odor, which limits palatability. With further distillation, methanol becomes practically odorless (Litovitz, 1990). Exposure to methanol can occur via inhalation, ingestion, and transcutaneous absorption.

Methanol (CH_3OH), which is hydrophilic (Yasugi et al., 1992), is distributed into the tissues proportional to the fraction of water in the tissue (Weiss et al., 1993). In a study by Leaf and Zatman (1952) on the absorption and elimination of methanol in humans ($n = 2$), it was concluded that exposure to a methanol vapor concentration of about 3000 ppm for 8 hours a day may cause toxicity. They suggested that the maximum concentration of methanol vapors to which workers may be safely exposed is 300 ppm. The current industrial threshold limit value (TLV) for methanol in industry is 200 ppm (260 mg/m^3) and provides a safety margin for a normal population. This standard is a time-weighted average for an 8-hour/day, 40-hour/week lifetime exposure (American Conference of Governmental Industrial Hygienists,

1990). Such an exposure would result in a methanol body burden of approximately 25 mg/kg (calculated as the product of methanol concentration in mg/m^3, duration of exposure, and ventilation rate divided by body weight) (Constantini, 1993). The TLV of 200 ppm was based on acute high-dose studies and not on chronic low level methanol exposure, and also did not include susceptible populations, such as fetuses or the elderly.

Methanol is an endogenous biomolecule. The sources of methanol are diet and natural metabolic processes. Methanol is available in the diet from eating fresh fruits and vegetables or from drinking fruit juices and aspartame sweeteners. Aspartame hydrolyzes in the gut, and 10% by weight is converted to free methanol that is available for absorption Methanol also is generated by methyltransferase enzyme system (Kavet and Nauss, 1990). Formate also is an endogenous biomolecule, and is a product of the metabolic degradation of several amino acids. Endogenous or background levels of formate in blood range from 0 to 20 mg/L (Baumann and Angerer, 1979). Exposure to methanol derived formate always must be evaluated in the context of normal endogenous levels (Kavet & Nauss, 1990).

Methanol Metabolism and Excretion

At low doses of methanol (smaller than 0.1 g/kg), 96.9% of the methanol is metabolized in the liver (Makar, Tephly, & Mannering, 1968), and 90% or more of the dose is excreted as CO_2 (Opperman, 1984); 2.5% is excreted through the pulmonary pathway; and 0.6% the clearance is through the renal pathway (Kavet & Nauss, 1990). In human and other primates, the hepatic enzyme, alcohol dehydrogenase, oxidizes methanol to formaldehyde. Formaldehyde, although it may be more toxic than its metabolites, does not accumulate during poisoning. The half-life of formaldehyde in blood is on the order of minutes (Litovitz, 1990). Formaldehyde, in turn, is metabolized to formic acid. This involves two processes: in the first reaction, formaldehyde is oxidized to S-formylgluthathione, involving formaldehyde dehydrogenase; and in the second, thiolase catalyzes the conversion of S-formylgluthatione to formic acid. Formic acid then dissociates into formate and hydrogen ions (Tephly & McMartin, 1984). The oxidation of formate to carbon dioxide occurs via a folate dependent system (Burkhart & Kulig, 1990).

In rats, the rate of elimination of methanol from the blood is dependent upon its level in the blood. At low levels, a first order disappearance occurs with a half-life of 2-3 hours. At higher blood methanol levels (300 mg/dl), disappearance from the blood obeys a zero-order kinetics with a variable half-life of about 27 hours (Horton, Wong, & Rickert, 1987; Kavet & Nauss, 1990).

Beverages containing ethanol drastically alter the metabolism of methanol because the same enzyme systems are responsible for the breakdown of both alcohols. Some variation in methanol concentration is due to the allelic diversity of alcohol dehydrogenase and aldehyde

dehydrogenase, the two critical enzyme systems that serve to metabolize methanol and ethanol. The metabolic rate constants of allelic variants differ substantially, and these genetic variations are common, and there is substantial association of genotype with racial types (Franzblau et al., 1992).

Methanol Toxicity in Humans

Virtually all of the available information on methanol toxicity in humans is related to the consequences of acute rather than chronic exposures. The characteristics of acute clinical methanol intoxication in humans consist of an initial phase of central nervous system depression, followed by a latent period of 12-24 hours after ingestion where few signs or symptoms are noted. Subsequently, there is a delayed phase of toxicity, which is characterized by metabolic acidosis and ocular damage (non-reactive pupils, retinal edema, and hyperemia of the optic disk), followed by blindness, coma, other central nervous system (CNS) signs, and eventually death (Røe, 1955). These late effects have been attributed to formate formation rather than to methanol, and are governed by the relative rates of formate generation and oxidation. These symptoms have been observed in both human and non-human primates. In experimental rodents, symptoms have developed only if the animals are made folate-deficient through dietary or biochemical modification. Currently, no data exist that permit the assessment of folate status on methanol toxicity in humans (MacGregor & Christensen, 1991; Tephly, 1991).

The metabolism of methanol gives rise to formic acid. This acid, which seems to account for methanol toxicity (Martin-Amat, McMartin, Hayreh, & Tephly, 1978), is an inhibitor of the mitochondrial cytochrome oxidase (the terminal member of the eukaryotic mitochondrial electron transport chain and an integral protein complex of the inner mitochondrial membrane) and may lead to histotoxic hypoxia (McMartin, Ambre, & Tephly, 1980; Nicholls, 1976; Tephly, 1991).

The body burden of formic acid in methanol poisoning is high enough to cause acidosis and other clinical symptoms. As formic acid accumulates and hypoxic metabolism occurs, acidosis causes dilatation of the cerebral vessels, facilitation of the entry of calcium ions into the cells. Formic acid concentrations in the kidneys may become sufficiently high to impede cyclic AMP-mediated parathyroid hormone-controlled Ca^{++} reabsorption in the distal tubuli (Savolainen, 1989).

The acute toxicity of methanol in humans has been recognized for a considerable time, with numerous episodes of poisoning documented since the late nineteenth century (Kavet & Nauss, 1990). McFarlan (1855) suggested that a mixture of one part of impure methanol (wood naphtha) to nine parts of ethanol constituted an inexpensive substitute for the use of ethyl alcohol in the manufacturing processes. Purification processes developed late in the nineteenth century improved the quality of methanol. As a result, there was an increase in the quantity of methanol mixed with ethanol and a

corresponding increase in toxicity after ingestion. In 1904, Wood and Buller reported on 235 cases of blindness and death due to ingestion to methanol (cited in Tephly, 1991). The largest incident of methanol poisoning in the United States occurred in Atlanta in 1951. Ninety gallons of 35-40% methanol-contaminated bootleg whiskey were distributed, causing 41 fatalities in 323 patients (Bennett, Cary, & Mitchell, 1953). In India, more than 300 deaths were reported in one mass methanol poisoning (Litovitz, 1990). As recently as December 1991, approximately 70 cases of methanol intoxication occurred in India, when nightclub owners substituted methanol for ethanol to reduce costs (Pamies, Sugar, Rives, & Herold, 1993).

In some cases, inhalation or dermal exposure to methanol has been implicated in workplace exposure. The maximum dose of methanol expected in EPA exposure scenarios is under 1 mg/kg of body weight (Cox, Baker, Kenedy, & Powers, 1987). A dose of 300 mg/kg of body weight (approximately 2 ounces for an average adult) is generally regarded as the lowest single lethal dose (Kavet & Nauss, 1990). Thus, acute toxic effects would not be expected from exposure to methanol vapor when used as a fuel.

In a study of teachers' aides who complained of physical ailments when exposed to vapors from direct-process duplicators, methanol concentrations ranged from 365 ppm to 3,080 ppm (mean concentration of 1,060 ppm) in the poorly ventilated rooms where the machines were located (Frederick, Schulte, & Apol 1984). Symptoms known to be associated with methanol toxicity, such as headaches, dizziness, blurred vision, and nausea, were reported significantly more often by aides than by teachers who were not as exposed. The incidence of symptoms was correlated with the amount of time the aides spent near the duplicators. A number of limitations were detected in this study. For example, symptom outcomes were self-reported and may have been compromised by response bias, the complaints were based on symptoms without accompanying clinical observations or any biological monitoring or neurobehavioral tests, and the possibility that other chemicals or solvents were responsible for the aides' complaints was not excluded. Even with these limitations, this study is unique among other epidemiological studies conducted on methanol exposure in that it provides data on ambient concentrations, duration of exposure, health status, and the relation between case attack rate and work-time exposed (Cox et al., 1987).

Neurologic Manifestations of Toxicity

Permanent motor impairment, consisting of tremors and limited painful movement of the limbs, has been reported in individuals ingesting near-fatal amounts of methanol (Guggenheim, Couch, & Weinberg, 1971). Furthermore, in a study by Phang, Passerini, Mielke, Berndt, & Garner King (1988), hemorrhagic necrosis in the basal ganglia, and hemorrhage into the ventricles of the brain were observed in 6 of 21 patients with methanol intoxication who had computed tomography (CT) brain scans. Also, autopsy studies on methanol poisoning victims have shown changes ranging from mild cerebral edema to large basal ganglia

hemorrhages. These reported injuries in central nervous system white and grey matter may explain neurologic syndromes seen after methanol poisoning. Syndromes include Parkinsonism, dyspraxia, pseudobulbar palsy, seizures, cognitive defects with myelopathy, and frontal release signs (Anderson, Shuaib, & Becker, 1989; Guggenheim et al., 1971; Mozaz, Wyke, & Indakoetxea, 1991; Phang et al., 1988).

Heparinization during hemodialysis for methanol toxicity, may contribute to the occurrence of the hemorrhage in the basal ganglia (Litovitz, 1990). Lesions in the cerebral hemispheres have been described on both computerized tomography and magnetic resonance imaging (MRI) scans (Chen, Schneiderman, & Wortzman, 1991), as well as lesions in the subcortical white matter, especially in the temporal and frontal lobes (Anderson, Shuaib, & Becker, 1987). Also, damage of the deep grey nuclei was shown by MRI in a patient with Parkisonism and dispraxia after acute severe methanol poisoning (Mozaz et al., 1991). By comparison, in alcoholics without clinical signs or symptoms of cerebral disorders, when examined by computerized tomography of the brain, changes, such as enlargement of the ventricular system appeared in patients with long history of heavy drinking, and widening of cortical sulci in young alcoholics (Bergman, 1984).

Because of the potential neurotoxic effects of methanol poisoning, it is appropriate to examine whether exposure to low concentrations of methanol vapor for sufficient periods of time may induce small, but significant, disturbances in central nervous system function. There is little information on this question. Two early accounts in the Soviet literature from different laboratories reported that several minutes exposure of humans by inhalation to very low concentrations of methanol (less than 12 mg/m^3) stimulated visual and peripheral olfactory receptors and may influence the processing of stimuli in the central nervous system (cited by Kavet & Nauss, 1990). Both of these studies are deficient in several respects: no description of the subjects was provided; several important details of the experimental procedure were not given; and no information was given regarding methanol purity and the analytical techniques used for determining methanol concentration.

In a pilot study, Cook et al. (1991) evaluated acute neurobehavioral effects of methanol vapor exposure at 192 ppm for 75 minutes in 12 healthy young males, who served as their own control. A battery of neurobehavioral tests assessed performance before, during, and after exposure. The effects were small and did not exceed the normal range.

Ocular Manifestations of Toxicity

The principal ocular lesion found in methanol poisoning is the development of toxic optic neuropathy. Examination of the optic nerve in detail (by light and electron microscopy) has shown swelling of the oligodendroglial cytoplasm in contact with the axons and of the astrocytes in the retrolaminar and the intraorbital optic nerves

(Hayreh, 1989).Murray et al. (1991) documented direct toxicity to the retina in a study of rodents treated with nitrous oxide and methanol. The most pronounced alteration was a severe vacuolation at the base of the photoreceptor outer segments and prominent expansion of the extracellular space at the bases of the pigment epithelium. In a control group treated only with methanol, subtle structural changes were noted in the mitochondria of the optic nerve. Naeser (1988) has implied that optic nerve damage from methanol poisoning is due to vascular disturbance through branches from a central artery extending perpendicular into the optic nerve providing generous perfusion, but Hayreh (1989) has argued that no such artery exists in humans.

Ocular toxicity has been described at length in the literature. Initially, when symptoms of visual disturbance are reported, hyperemia of the optic disc is found, followed by peripapillary edema. Ultimately, the optic nerve becomes swollen, in conjunction with the retinal vasculature. This edema may persist for a substantial period of time, and if damage is sufficiently severe, atrophy of the optic nerve ultimately results (Gilger & Potts, 1955; Hayreh et al., 1977). There is the risk of total blindness, even though a patient may recover from metabolic acidosis (Tephly, 1991). The lack of a relationship between methanol concentration and the development of ocular toxicity and metabolic acidosis implies that methanol metabolites may be responsible for the injury.

At present, the specific role of formic acid in the development of ocular toxicity is unclear. Some authors believe that formic acid inhibits cytochrome oxidase in the fundi, and also disrupts the flow of axoplasm (Nicholls, 1976). Others (cited Winchester, 1990), have suggested that the local production of formaldehyde from methanol in the retina is responsible for the production of optic papillitis and retinal edema, and subsequent blindness. In addition, metabolic acidosis could independently lead to the development of visual problems, such as photophobia and blurred or indistinct vision, hyperemia of the disc, sluggishly reactive and dilated pupils, and even blindness (McCormick, 1990). Decreased pupillary response to light has prognostic significance. Patients who maintain normally reactive pupils do not experience permanent visual loss after methanol poisoning (Benton & Calhoun, 1952).

Other Toxic Effects of Methanol

Hemorrhagic gastritis has been described in fatal methanol ingestions, as well as acute congestion of the kidneys and liver (Phang et al., 1988). Hyperamylasemia frequently has been noted and is usually ascribed to the development of acute pancreatitis (Hall, 1991). Other complications of methanol poisoning are reversible cardiac failure, with cardiomegaly, pulmonary edema, and electrocardiogram (ECG) changes of stable bifasicular block; self-limited atrial fibrillation; and diffuse T-wave abnormalities (Cavalli, Volpi, Maggioni, Tusa, & De Pieri, 1987). Developmental and reproductive toxicity of methanol are of concern, and may include birth defects, motor behavior problems, and attention deficit

disorders (Weiss et al., 1993).

Methanol Toxicity in Non-Human Primates

Monkeys exhibit features of methanol poisoning syndrome as presented in humans. These include the development of metabolic acidosis coincident with the production of formic acid; severe optic disc edema associated with marked intra-axonal swelling; mitochondrial disruption; dysfunction of the optic nerve pathway and slight central nervous system depression, followed by a metabolic acidosis occurring up to 12-24 hours later. This is followed by deterioration characterized by anorexia, vomiting, weakness, hyperpnea, and tachypnea. Experimental animals have died of respiratory failure 20-30 hours following methanol administration (McMartin, Makar, Martin-Amat, Palese, & Tephly, 1975). A similar study by Hayreh et al. (1977) provides a model for ocular toxicity. Attenuated and prolonged intoxication was produced by repetitive administration of methanol. Animals developed prolonged formic acidemia, metabolic acidosis, and ocular toxicity 40-60 hours following the initial dose.

Gilger and Potts (1955) were the first researchers to demonstrate ocular toxicity and metabolic acidosis in monkeys following the administration of methanol. They reported a minimum lethal dose of 3 g/kg body weight for rhesus monkeys. Clinically, the signs observed in the monkeys were similar to those observed in humans. The depletion of bicarbonate in methanol-poisoned monkeys has been shown to occur in a mole-for-mole fashion with the accumulation of formic acid (Clay, Murphy, & Waykins, 1975; Tephly, 1991). Apparently, the buffering capacity of blood and tissues is maintained during the latent period. The blood pH is not significantly decreased, because the latent period represents compensated metabolic acidosis.

In other studies, researchers have demonstrated that folate-deficient monkeys became especially susceptible to the toxicity of methanol relative to the amount of formate produced (McMartin, Martin-Amat, Makar, & Tephly, 1977). Others have indicated that methanol toxicity, once established in the monkey, can be reversed with 5-formyltetrahydrofolic acid administration, by stimulating the rate of formate oxidation or utilization (Noker, & Tephly, 1980).

Knowledge of which metabolic system is responsible for the oxidation of methanol is relevant for the selection of appropriate inhibitors that would prevent or delay the onset of toxic symptoms produced by this alcohol (Tephly, 1991). Humans and monkeys possess low hepatic H_4 folate levels, low rates of formic acid oxidation, and accumulation of formic acid after methanol exposure. In addition to low hepatic H_4 folate concentration, monkeys and humans also have low hepatic 10-formyl H_4 folate dehydrogenase levels, the enzyme that is the ultimate catalyst for conversion of formic acid to carbon dioxide (Johlin, Fortman, Nghiem, & Tephly, 1987; Tephly, 1991). The rate of formate oxidation is related to hepatic tetrahydrofolate (H_4 folate) content and the activities of folate-dependent enzyme. In the human

liver, H_4 folate levels were only 50% of those observed in a rat liver and similar to those found in a monkey liver. Total folate also has been found to be lower (60% decreased) in a human liver than in rat or monkey livers (Johlin et al., 1987). A mouse liver contains much higher hepatic H_4 folate and total folate than a rat or monkey liver, which is consistent with higher formate oxidation rates in this species.

Formaldehyde accumulation has not been detected in body fluids or tissue after methanol administration. Following intravenous infusion of formaldehyde in monkeys, clearance of this compound from the blood occurred rapidly ($t_{1/2}$ 1.5 min) (McMartin, Martin-Amat, Noker, & Tephly, 1979). This means that formaldehyde may not be responsible for the health effects following methanol exposure and that the metabolite formaldehyde may not be a preferred biological marker of exposure.

Methanol Toxicity in Non-Primates and Other Considerations

Although humans are susceptible to methanol poisoning, lower species, such as rats or mice, are not (Tephly, 1991). The effects of methanol in lower animals are quite different from those in humans and primates in that metabolic acidosis and ocular toxicity are normally not found (Makar & Tephly, 1977; Røe, 1982; Tephly, 1991). McMartin et al. (1977) demonstrated that formic acid at any dose is metabolized to carbon dioxide in monkeys at rates much slower than those in rats. Thus, it is inappropriate to extrapolate results obtained from experiments using non-primate animals to humans.

The first step in the metabolic pathway of methanol is its oxidation to formaldehyde. In rats, a catalase-peroxidase system is primarily responsible for this initial step, while in humans and monkeys, alcohol dehydrogenase plays this role (Tephly & McMartin, 1984). Humans and monkeys do not oxidize methanol through a catalase-dependent system because of the low activities of peroxide-generating enzymes (urate oxidase, glycolate oxidase, and xanthine oxidase) (Goodman & Tephly, 1970). Another possible pathway in methanol oxidation is the hepatic microsomal mixed-function oxidase system. The rate of formaldehyde production is approximately the same in the different species (Tephly & McMartin, 1984).

Non-primate species are ordinarily resistant to the accumulation of formate and its associated metabolic and visual toxicity. Rats restricted to a folate-deficient diet for 10-12 weeks, however, showed a marked decrease in formate oxidation and a marked sensitivity to methanol poisoning, as evidenced by high blood formate levels and marked decreases in blood pH (Makar & Tephly, 1977). The pig has been proposed as a potential animal model because of its low liver tetrahydrofolate levels and slower rates of formate metabolism, but Dorman et al. (1993) concluded that female minipigs do not appear to be a suitable animal model for acute methanol-induced toxicity. Following a gavage of 5 g/kg of methanol or intravenous formate of 425 mg /kg, doses high enough to cause neuro-ocular toxicity in

primates (Martin-Amat et al., 1978), the minipigs did not develop optic nerve lesions, toxicologically significant formate accumulation, or metabolic acidosis.

A non-primate model of methanol-induced visual toxicity has been developed using rats treated with subanesthetic concentrations of nitrous oxide (which blocks methionine synthetase by inactivating vitamin B-12, which disrupts the normal pathways of folate metabolism) to inhibit the oxidation of formic acid, methanol's toxic metabolite (Eells, Black, Tedford, & Tephly, 1983). When pre-exposed to nitrous oxide, methanol-intoxicated rats developed the following within 36 hours of methanol administration: formic acidemia, metabolic acidosis, and visual toxicity analogous to the methanol poisoning syndrome in humans. Alterations in electroretinography occurred at formate concentrations lower than those associated with other visual changes and provided functional evidence of direct retinal toxicity in methanol poisoning (Eells, 1991; Murray et al., 1991).

The susceptibility of rodents to methanol toxicity is different than primates, but some research had ignored this assumption in their experimental designs. For example, Maejima et al. (1992) evaluated the toxicity in rats of methanol exhaust generated by methanol with a 15% gasoline fueled engine in three different concentrations for a maximum of 28 days. This exposure to exhaust containing other components, such as carbon monoxide, formaldehyde, and hydrocarbons was an appropriate imitation of the real environment, but one must ask how such findings can be extrapolated to humans if the detoxification process is different in rodents.

A non-primate laboratory animal model also should be developed that metabolizes methanol in a manner analogous to that in humans. Although in several different laboratory animal models, in which formate oxidation has been compromised by various experimental means, a laboratory animal model is needed in which the actual enzymes are kinetically similar to those in humans. The availability of such an animal model is especially important for studies of chronic exposure to low levels of methanol. The difficulty is that the manipulations that produce folate deficiency in laboratory rodents, such as maintenance on very low folate diets supplemented with sulfa drugs or administration of nitrous oxide, are likely to confound outcomes in chronic exposure studies (MacGregor & Christensen, 1991).

Developmental and Reproduction Toxicity in Rodents

One of the most important concerns about the toxicity of a chemical is its effect on fetus development and reproduction. The developmental health risk to the fetus from methanol exposure during pregnancy is not known (Bolon, Dorman, Janszen, Morgan, & Welsch, 1993), but a wide range of dose-dependent teratogenic effects in rodent embryos has been investigated recently.

Bolon et al. (1993) found that pregnant mice exposed to methanol

inhalation at high levels (10,000-15,000 ppm) induces concentration depended developmental toxicity, and the spectrum of teratogenic effects depended upon the stage of embryonic development and the number of methanol exposures. Terata included neural and ocular defects, cleft palate, hydronephrosis, deformed tails, and limb abnormalities. Females had a twofold higher incidence of neural tube defects and cleft palate, while males were more than twofold likely to have hydronephrosis. In a study by Rogers et al. (1994), exposure of pregnant mice to methanol for 7 hr/day on gestation day 5-8 produced cleft palate, and skeletal anomalies, but exposures after gestation day 9 caused no developmental toxicity. The no observed adverse effect level (NOAEL) for developmental toxicity in this study was 1000 ppm, based on an increased incidence of cervical ribs at 2000 ppm.

Similar teratogenic effects were found in mice offsprings after maternal ethanol consumption during gestation. The anomalies included limb defects, hydrocephalus and exencephaly, hydronephrosis and hydroureter, undescendent testicles, and cardiovascular defects (Randall and Taylor, 1979; Gage and Sulik, 1991). Malformations due to ethanol for experimental rodents and in human populations suggest that parallels for methanol might also be seen (Jones and Smith, 1973). Although, maternal malnutrition or other environmental confounders, associated with chronic alcohol use, must be taken into consideration.

In a study by Nelson, et al. (1985), the teratogenic effects of methanol and ethanol at high inhalation levels in rats were evaluated. They demonstrated that methanol administered at 20,000 ppm was teratogenic, and possible teratogenic at 10,000 ppm. The congenital malformations were extra or rudimentary cervical ribs and urinary or cardiovascular defects. In contrast, 20,000 ppm ethanol was only possibly teratogenic. In another study by Weiss, et al. (1994) pregnant rats, beginning on gestational day 6, animals were exposed to 4,500 ppm methanol vapor for 6 hour daily and continued exposing both dams and pups until postnatal day 21. None of the behavioral endpoints examined distinguished methanol exposed from control offspring, but the blood methanol determinations were about twice the levels in pups than the levels measured in their dams.

The potential toxic effects of methanol vapors on testicular production of testosterone and the morphology of testes have been investigated in normal or methanol-susceptible folate-reduced rats. The data indicate that low-level methanol may not cause an inhibitory effect on testosterone synthesis (Lee, Brady, Brabec, & Fabel, 1991).

Treatment of Methanol Poisoning

Adequate recognition of methanol poisoning and treatment is essential to patient survival. Treatment delays beyond 8 to 10 hours and a blood formic acid above 50 mg/dl are predictive of severe methanol poisoning, possibly leading to permanent sequelae (Anderson et al., 1989; Mahieu, Hassoun, & Lauwerys, 1989).

As with many acute intoxications, it is necessary to assure airway patency and adequacy of respirations and oxygenation, especially if CNS or respiratory depression are present. Where there is direct contact with the skin, removal of clothing and washing of the patient also are recommended. Irrigation of the eye with normal saline or tepid water is indicated if the eyes have been splashed or come into contact with methanol (Hall, 1991).

Standard treatment for methanol toxicity consists of induced emesis or lavage, charcoal for co-ingested toxins, intravenous bicarbonate for the acidosis, administration of ethanol to inhibit conversion of methanol to formic acid via alcohol dehydrogenase, fluid replacement for dehydration, folate to enhance formic acid oxidation, and hemodialysis to remove methanol and formate (Anderson et al., 1989; Becker, 1983; Ekins, Rollins, Duffy, & Gregory, 1985; Osterloh, Pond, Grady, & Becker, 1986). In humans, early or prolonged hemodialysis has been responsible for recovery without sequelae, even when initial blood methanol levels were very elevated (Hall, 1991). Peritoneal dialysis is not appropriate management since it is only one eighth as effective as hemodialysis (Litovitz, 1990).

Ethanol is a specific antidote for methanol poisoning, which acts by competitive inhibition of alcohol dehydrogenase. Hepatic alcohol dehydrogenase has a much greater affinity (10-20 times) for ethanol (Hall, 1991). 4-Methylpyrazole (4-MP), an inhibitor of alcohol dehydrogenase, may also be useful for the treatment of methanol and ethylene glycol intoxications. A mild, transient increase in liver enzymes might be observed in some subjects treated with multiple doses of 4-MP. Nevertheless, the slower elimination rate and lesser degree of toxicity of 4-MP, its longer duration of action, greater ease of dose titration, and the absence of CNS depressant activity make it preferable to ethanol in therapy. 4-MP is not available for clinical use at this time (Litovitz, 1990). In non-human primates, administration of folinic acid (Leukovorin) or folic acid enhances conversion of the toxic formate metabolite to carbon dioxide and water (Hall, 1991).

Occupational Exposure and Biological Monitoring of Methanol

The lack of measured exposure to a given chemical or physical hazard is a recurring problem in occupational epidemiology because many diseases have long latencies and occupational hygiene records are often unavailable from the period of onset or at all. In recent years, however, biological monitoring has become available for an increasing range of toxic exposures (Osterloh & Tarcher, 1992). Biological monitoring is the measurement of a chemical, its metabolite, or a nonadverse biochemical effect in a biologic specimen for the purpose of assessing exposure (Lauwerys, 1983).

Methanol and formic acid are normal trace constituents of body fluids, so biological monitoring of methanol has the added problem of distinguishing a normal from an elevated level, and not just

identifying the presence of a foreign compound (Franzblau et al., 1992). The variability of formic acid levels in blood and urine is very high since it is synthesized in various metabolic pathways or from alimentary uptake (Baumann & Angerer, 1979). While serum formate concentrations correspond more to the severity of the clinical condition (ocular signs and acidosis) than methanol concentrations during overdosage (Osterloh et al., 1986), determinations of formic acid in blood or urine may not be sufficient to estimate the degree of exposure.

Baumann and Angerer (1979) studied the validity of blood and urine formate concentrations in 20 print shop workers exposed to methanol before and after their workshift and in a control group of 51 workers who had no contact with methanol. In the exposed workers, methanol concentration in the alveolar air was determined. Methanol concentration at three sites in the print shop were 85, 101, and 134 ppm respectively. Methanol concentration in the alveolar air and formic acid concentration in the blood and urine were determined at the beginning and at the end of the shift. For comparison, formic acid concentrations in the blood and in urine were determined at corresponding times of the day in two groups of 36 and 15 subjects who had no contact with methanol. Formate concentration in the blood rose an average of 4.7 µg/mL over the course of a day (mean 3.2 µg/mL, SD = ± 2.4 before the work shift to mean 7.9 µg/mL, SD = ± 3.2 when work ended). The pre- and post-exposure differences in blood formate concentrations were statistically significant and every worker registered an increase. These positive changes may be explained by the variability in methanol air concentrations at the print shop that ranged from 6 to 677 ppm, possible additional dermal exposures resulting from intentional or unintentional contact with methanol in the ink or with methanol as a solvent, increased respiratory exposure due to workload, and/or dietary changes. In this study , the researchers were not able to find a correlation between methanol concentration in the environment and the biological monitoring, such as formate in blood and urine. The level of blood formate reached at the end of the workshift was not higher than the levels considered to be endogenous formate concentration.

Lee, Terzo, D'Arcy, Gross, and Schreck (1992) investigated whether formate accumulates in the blood of humans under carefully controlled exposure to methanol with dietary restrictions to avoid food that would raise formate levels. Six healthy male subjects between the ages of 29 to 55 years were exposed to 200 ppm methanol in a monitored chamber for 6 hours, and blood methanol concentrations determined under different physical exercise conditions. Formate did not accumulate in the blood at air levels below 200 ppm, and blood concentrations of methanol were the same regardless of whether workers were engaged in light physical activity when they were exposed to methanol vapors up to 200 ppm. Even though the study was fairly well-designed, the sample size is too small to draw any concrete conclusions.

Liesivuori and Sovalainen (1987), found that the output of urinary

formate was linearly proportional to the methanol concentration (40-160 ppm) in the air,but only at 16 hours after exposure. No correlations were found between methanol exposure and urinary formic acid or methanol concentration immediately after exposure. In occupational settings, they recommend collecting samples of urine formic acid on the last day of the week to allow formic acid to build up. In this study the sample size was small (n = 13) and it lacked any monitoring of skin absorption (only methanol in the air was measured). Franzblau, Levine, Schreck, D'Arcy, and Qu, (1992) exposed four human subjects to approximately 200 ppm of methanol in a test chamber for 6 hours. The ambient air in the chamber was monitored for methanol and urine was monitored for formic acid. Urine specimens were collected immediately before, immediately after, and 16 hours following exposure. Mean urinary formic acid increased, though not significantly, from baseline at the end of the exposure session, but had returned to baseline in samples collected 16 hours after exposure. These results contradicted those of Liesivuori and Sovolainen, that the measurement of urinary formate in specimens collected 16 hours after cessation of exposure to methanol may not be appropriate in the biological assessment of methanol exposure.

Another study (Yasugi et al., 1992) compared formate excretion with methanol excretion in the urine of workers occupationally exposed to methanol and concluded that urinary formic acid was less sensitive than urinary methanol as a marker of methanol vapor exposure. Urinary methanol concentration also has been found to depend strictly on the duration and intensity of methanol exposure, which suggests that measurement of urinary methanol concentration would be a reliable parameter for evaluating the degree of methanol exposure (Ferry, Temple, & McQueen, 1980).

In another study by Sedivec, Mraz, & Flek (1981), five volunteers were exposed to constant and suitably graded concentrations of methanol vapor for a period of 8 hours. From the concentration in the inspired air, lung retention, minute lung ventilation, and duration of exposure, a calculation of the retained methanol dose was performed. It correlated well with the methanol concentration (mmol/L or mg/L) collected in the urine during the shift. It was suggested that urinary methanol concentration would be a reliable parameter for evaluating the degree of methanol exposure, even if the subjects performed physical work and, consequently, had enhanced lung ventilation, or in cases where the air also contained other organic solvent vapors. The limitations of this study are that its conclusions may be different in actual occupational settings where methanol concentrations fluctuate and exposure is interrupted by work breaks. Also, the sample size was quite small and cannot be generalized.

All the studies in biological monitoring presented above lack at least one of the following: a control group; a sufficient sample size; maintenance of a constant or known exposure; randomization; blinding of the subject, blinding of the researcher or both; dietary safeguards; or valid biological monitoring. These research studies

still are valuable, however, for further research and for hypothesis formulation.

Summary of Methanol Toxicity

Methanol is a toxic chemical that may eventually be introduced into the environment as a fuel, in which case a large population would be exposed. The acute toxicity of methanol is characterized by central nervous system depression, metabolic acidosis, ocular damage, coma, and death.

The effects of methanol in rodents are different from those found in humans and primates because metabolic acidosis and ocular toxicity are not noted after high-dose exposures, probably because rodents have higher hepatic H_4folate and total folate than humans. This is consistent with the higher formate oxidation rates in this species.

The recommended treatment of methanol poisoning is based on clinical signs, presence of metabolic acidosis, and methanol blood levels. Treatment includes induced emesis and lavage, administration of ethanol, folate, and hemodialysis.

Methanol in blood or urine appear to be suitable tests for the biological monitoring of methanol-exposed groups. Formate levels seem to be not useful in monitoring methanol low level exposures, but suitable in acute high level exposures as markers of toxicity. Methanol is a suitable monitor in low level exposures, and less useful as a marker of toxicity.

Susceptibility and Folate Deficiency

Variation exists in the susceptibility of individuals to exposure. Because humans are genetically and environmentally heterogeneous, the identification of individuals at risk for significant exposure based on susceptibility may permit more precise targeted surveillance of humans at high risk than surveillance of entire populations. A susceptible person is defined as one who develops a disease if there is exposure whereas the majority of the population does not develop the disease if they are exposed to the same level. Susceptibility typically implies that the individual has biological characteristics that may lead to a different biological response. Analysis of these contributors to variability reveals important aspects of the intrinsic mechanism of defense, adaptation, and homeostasis, all of which influence toxic effects (Guidotti, 1992).

Thresholds are among the most controversial concepts in toxicology. The concept of a threshold implies that there is some dose of a compound that is not capable to produce a detectable adverse effect, although exposures below a certain threshold are not necessarily innocuous and a large part of the exposure-response relationship is hidden. The threshold for a heterogeneous population may be impossible to define since the resultant curve is composed of many thresholds, each at different doses (Sheehan, Young, Slikker,

Gaylor, & Mattisson, 1989). An example of this is gender differences in response to toxic substances. This is of particular importance given the influx of women into traditionally male-dominated occupations. In fact, most workplace health standards tend to be based on criteria derived from an assessment of how men have responded historically to pollutants (Calabrese, 1978).

The factors that can influence an individual's susceptibility to chemical exposure in the workplace and the general environment are quite varied and include genetic background; age; gender; nutritional status; physiological status, including pregnancy; presence of disease; exercise; lactation; behavioral or lifestyle considerations and experiences; sunlight; barometric pressure; immunization; levels of exposures in the workplace and environment; drugs; circadian and seasonal variations; dietary factors; cardiovascular, liver, renal, gastrointestinal and immunological function; stress; fever; alcohol intake; smoking; and water content in the body (Tarcher & Calabrese, 1992). All of these factors can be categorized as contributors to human biologic variation.

The scientific evidence of human susceptibility to methanol toxicity has not been demonstrated, but experimental research in rodents and primates give evidence for a role of folate-dependent formate metabolism. For example, after methanol administration to monkeys, having lower hepatic folate levels than rats, treatment of either 5-formyltetrahydrofolic acid or folic acid results in a decrease of formate accumulation, absence of metabolic acidosis and no blood bicarbonate depletion (Noker & Tephly, 1980;see review above).

Folate Deficiency

Folate is a generic descriptor for a group of compounds that have nutritional properties and chemical structures similar to those of folic acid, which is essential in the synthesis of new cells. Folic acid deficiency can lead to impaired cell division, alterations in protein synthesis, DNA damage, and other metabolic effects, which are most immediately observed in rapidly dividing cells (MacGregor & Christensen, 1991).

The apparent variability of humans to methanol exposure may have several explanations (Tephly, 1991). Røe (1955) proposed that the variability in reaction of humans to methanol could be explained by the concomitant ingestion of ethanol and methanol. Recent research has suggested another possible explanation for individual variation. Nutritional differences, such as folate deficiency, may exist among individuals and folate status may be a key factor in determining individual variability to methanol toxicity.

In Germany, 15% of different age groups are said to experience folate deficiency (Hages, Jenke, Mirgel, & Pietrzik, 1989). In the United States, data collected by the second National Health and Nutrition survey (NHANES II) from 1976-1980 indicated that 13% of

males and 12% of females had low serum folate levels, and red blood cell (RBC) folate levels were low in approximately 10% of apparently healthy male and female subjects (cited in Senti & Pilch, 1985). In a national nutrition survey conducted in Canada in 1970-1972 involving 12,000 subjects of all ages, there was 10% folate deficiency present in children and adolescents; and in 25% of children and 40% of Eskimo adolescent; and in the high-risk groups an average of 25% were folate deficient as assessed through serum levels (MacGregor & Christensen, 1991). Based on the recommended dietary allowance (RDA) of 400 micrograms/day, the results of one study indicated that folate intake in the United States is low, particularly among women and blacks (Subar, Block, & James, 1989).

The folate-deficient populations that may be more susceptible to the effects of methanol/formate toxicity are defined by pregnancy, diet, alcohol intake, malabsorption, and medication usage.

Folate Deficiency and Pregnancy

Pregnancy is a prime cause of folate deficiency (expressed as megaloblastic anemia) in human population, and as many as 20% of all pregnant women who do not receive folate supplements are marginally-to-severely folate deficient (MacGregor & Christensen, 1991). In pregnancy, there is a much higher risk of developing folate deficiency due to elevated folate catabolism (McNulty, McPartlin, Weir, & Scott, 1993). This is primarily due to the folate requirement for DNA synthesis of fetal and placental rapidly growing tissue, and even an ideal diet cannot provide sufficient folate during pregnancy (Hages et al., 1989). In a series of non-randomized and randomized intervention trials and case-control and cohort studies, however, women using multivitamins or folic acid supplements during the first 6 weeks of pregnancy experienced a three-to-four-fold reduction in neural tube defects in their offspring. The amounts of folic acid contained in multivitamins (usually 200-400 mcg per day) appear adequate to greatly reduce, and even eliminate, excess risk of folate-related neural tube defect (Willett, 1992).

Folate Deficiency and Adolescence

Folate status also may be compromised during adolescence (Bailey, 1990). Tsui and Nordstron (1990) found evidence of low folate status in a bi-racial sample of 164 subjects of adolescence (aged 12-15 years) in three junior high schools in metropolitan Kansas City. Thirteen percent of the boys and 40% of the girls were folate-deficient (as judged by amount of erythrocyte folate < 140 ng/mL), although no racial differences were observed.

Folate Deficiency and Psychiatric Disorders

The incidence of folate deficiency is high in patients with psychiatric disorders, including depression, dementia and schizophrenia. The cause for this deficiency is unknown, but inadequate dietary intake probably plays some role (Young &

Ghadirian, 1989). The diminished absorption of folate from the gastrointestinal tract can also be caused by antipsychotic drugs such as the phenothiazines (Farrell, Jamjoon, Donaldson, & Dickerson, 1988).

Psychiatric symptoms occur more frequently with folate deficiency, although among psychiatric patients, these symptoms are less severe in patients whose levels of folic acid are normal. Folic acid deficiency lowers brain S-adenosylmethionine, which has antidepressant properties, and 5-hydroxytryptamine (5HT). S-Adenosylmethionine also raises brain 5HT, and depression associated with folate deficiency is probably related to low amounts of this chemical (Young & Ghadirian, 1989).

Because of its deficiency in psychiatric patients, folic acid should be given as an adjunct in the treatment of patients with uni-polar or bi-polar affective disorders or anorexia, geriatric patients with mental symptoms (e.g. senile or arteriosclerosis dementia), and patients with gastrointestinal disorders who exhibit psychiatric symptoms. Although the majority of these patients probably will not markedly improve by folic acid therapy, a significant minority are likely to have some folate-responsive symptoms (Young & Ghadirian, 1989).

Folate Deficiency and Alcoholism

The disruption in folate metabolism that occurs with the ingestion of ethanol by alcoholics is probably related to a variety of factors, including an inadequate diet, the interference of folate transport to tissues and intestinal absorption, increased excretion of folate, impaired formation and/or hydrolysis of polyglutamates, and possible folate destruction caused by intermediates generated from ethanol metabolism (Koblin & Everman, 1991).

Schwab and Powers (1992) assessed the prevalence of folate deficiency in emergency department patients with alcohol-related illness or injury, and found that the prevalence of folate deficiency was low and did not differ from the general emergency department population.

Folate Deficiency and the Elderly

Folates exist in foods primarily as pteroylpolyglutamates that must undergo hydrolysis to pteroylglutamate (monoglutamates) by the small intestine in order to be absorbed. Luminal folylpolyglutamate conjugase deficiency causes malabsorption of folate and subsequent folate deficiency in older rats, a phenomenon that may be related to the high prevalence of folate deficiency in the elderly (MacGregor & Christensen, 1991). In a study evaluating the nutritional status of the elderly in Spain, although vitamin B12 intake and blood levels were satisfactory, folate intake was below the recommended amount in 41.6% of the cases, and serum and RBC folate levels (115.37 ± 36.7 ng/ml) were deficient in 80% of the 72 non-institutionalized elderly

(Ortega, Redondo, Andres, & Eguileor, 1993).

Folate Deficiency and Malabsorption

Patients with inflammatory bowel disease (IBD) also are at risk for folate deficiency. Despite the fact that folate is absorbed principally in the jejunum and that IBD primarily affects the terminal ileum, colon, and rectum, folate malabsorption does occur in a sizable proportion of IBD cases (Clinical Nutrition Cases, 1988).

Jejunoileal bypass, gastroplasty, and gastric bypass are all surgical procedures for the treatment of morbid obesity. Jejunoileal bypass produces malabsorption of folate by bypassing a major portion of the absorptive surface of the small intestine (Hocking, Duerson, O'Leary, & Woodward, 1983). In gastric bypass, the stomach is stapled just distal to the cardia. Low plasma folate levels have been noted in 42% of a group of gastric bypass patients after a mean period of 3 years (Halverson, 1986). In gastroplasty, the stomach is stapled, with a small opening left to permit the normal passage of food into the distal stomach and duodenum. Gastroplasty poses a risk of long-term malnutrition due to diminished food intake or to vomiting from either gorging or gastric-outlet obstruction (Sugerman & Wolper, 1984).

Folate Deficiency and Medication

Folate deficiency often occurs in individuals with epilepsy taking anticonvulsants, because anticonvulsants inhibit folate absorption. In these patients, folate deficiency may be associated with psychiatric symptoms (Young & Ghadirian, 1989). Folic acid levels during pregnancy and pre-pregnancy were determined in 51 mothers with epilepsy and those for matched control. In all study periods, the serum folic acid levels of mothers with epilepsy were significantly lower than those of the control (Ogawa, Kaneko, Otani & Fukushima, 1991).

Folic acid should be administered with caution to drug-treated patients with epilepsy because the ability to control seizures may be affected (Butterworth & Tamura, 1989). A significant inverse correlation was observed between serum levels of folates and phenytoin, which suggests an adverse effect of the drug (Penarrocha, Rodriguez, Giner & Sirvent, 1989).

Methotrexate (MTX) and its less active metabolite, 7-OH-methotrexate, are transported intracellularly by an active transport mechanism and accumulate within the cell in a polyglutamated form. A 7-hydroxy derivative is produced, at least in part, in hepatic cells and is formed most effectively following medium- and high-dose MTX therapy. In cancer patients, MTX and its polyglutamates act intracellularly by inhibiting the enzyme dihydrofolate reductase (Furst & Kremer, 1988).

Methotrexate also has been used in the treatment of psoriasis, a

non-neoplastic disease of the skin characterized by abnormally rapid proliferation of epidermal cells (Nichols, 1993). In psoriatic patients on long-term treatment with MTX, folate may decrease (Zachariae, Schroder, Foged & Sogaard, 1987).

Methotrexate is effective, as well, in the treatment of refractory rheumatoid arthritis, although the mechanism of action of MTX in both rheumatoid arthritis and psoriasis remains unknown, it is unclear whether MTX acts primarily as an anti-metabolic drug, an immunosuppressive drug, an anti-inflammatory agent, or a combination of the three (Furst & Kremer, 1988).

Sulfasalazine, which has been shown to be effective in the treatment of active Crohn's disease and ulcerative colitis, inhibits folic acid absorption, interferes with folic acid metabolism, and may result in decreased serum folic acid concentrations and even possible folic acid deficiency in some patients. Several mechanisms appear to be involved, including inhibition of hepatic folate metabolism, intestinal transport of folic acid, and jejunal brush-border enzyme folate conjugase, which hydrolyzes polyglutameal folate to the monoglutamate form for intestinal transport (Peppercorn, 1984).

Franklin and Rosenberg (1973) have shown that the malabsorption of folate is not simply another facet of jejunal dysfunction in IBD, but that malabsorption is worsened by sulfasalazine, which is very effective in combating the inflammation associated with this disease. They also discovered that orally administered sulfasalazine interfered with folic acid absorption both in patients with IBD and in normal subjects. Sulfasalazine is a competitive inhibitor of brush border conjugase, and inhibits the hydrolysis of polyglutamyl and monoglutamyl folate. Sulfasalazine may act as an inhibitor of dihydrofolate reductase, and folate absorption (Halasz, 1990), and folate malabsorption and deficiency are predictable in most patients using sulfasalazine (Halsted, Gandhi & Tamura, 1981).

Summary of Folate Deficiency

Several population groups are at high risk for folate deficiency; they include pregnant women, the elderly, adults and adolescents of low economic status, individuals with chronic inflammatory bowel disease, patients using anticonvulsants or antibiotics, those in chemotherapy, individuals with mental and psychiatric disorders, and alcoholics. These individuals are potentially at high risk from methanol exposure.

No information was found in the published literature on the toxicity or metabolism of methanol in folate-deficient humans, and no control studies have been conducted to determine how much of the human susceptibility to methanol may be apparently attributable to differences in folate status (MacGregor & Christensen, 1991).

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Table 1. Test Procedures

For the First Meeting	For the Experimental Sessions		
	Preexposure activity	Exposure activity	Postexposure activity
* Study explanation * Consent form and Bill of Rights * Questionnaire * Physical examination * Folate levels in plasma/RBC * NBT tests: Symbol Digit, Sternberg, 2 & 7, Stroop * Visual tests: Vistech & Lanthony * Neurophysiological test: P-300	* 24-hour diet: No fruit or fruit juice, alcohol, vegetables, coffee, medicines, aspartame * NBT tests: Symbol Digit, Sternberg, 2 & 7, Stroop * Visual tests: Vistech & Lanthony * Neurophysiological test: P-300 * Urine sample and measure * Blood sample	* 4 hours exposure to 200 ppm methanol vapors or water vapors * Ten blood samples: first hour every 15 minutes and the other three, every 30 minutes * NBT tests: 2 & 7, Stroop, Symbol Digit (last 30 minutes of exposure).	* Urine collected immediately after exposure and 4 hours later * Blood samples every hour during 4 hours * Immediately after exposure: Vistech and Lanthony * Immediately after exposure: P-300 * Immediately after exposure: Sternberg * At the end of second session: exposure guess

Table 2 Description of Study Subjects

Variable	Female	Male	Total
Age(years), n (%)			
26-38	5 (45.5)	13 (86.7)	18 (69.2)
39-51	6 (54.5)	2 (13.3)	8 (30.8)
Mean ± SD	39.6 ± 6.9	33.0 ± 5.7	35.7 ± 6.8
Education, n (%)			
High School	1 (9.1)	3 (20)	4 (15.4)
College Trade School	6 (54.6)	9 (60)	15 (57.7)
Graduate School	4 (36.3)	3 (20)	7 (26.9)
Race, n (%)			
Hispanic Latino	5 (45.5)	0 (0)	5 (19.2)
American Indian	0 (0)	1 (6.7)	1 (3.9)
Caucasian	6 (54.5)	12 (80)	18 (69.2)
Asian	0 (0)	2 (13.3)	2 (7.7)
Weight (lbs), n (%)			
110 - 144	5 (45.4)	1 (6.7)	6 (23.1)
145 - 179	3 (27.3)	7 (46.7)	10 (38.4)
180 - 214	3 (27.3)	5 (33.3)	8 (30.8)
215 +	0 (0)	2 (13.3)	2 (7.7)
Mean ± SD	151.3 ± 20.7	185.0 ± 31.7	172 ± 34
Height (inches), n (%)			
59 - 65	5 (45.4)		5 (19.2)
66 - 72	6 (54.6)	14 (93.3)	20 (76.9)
73 +	0 (0)	1 (6.7)	1 (3.9)
Mean ± SD	65.6 ± 3.6	72.5 ± 2.4	69.6 ± 4.5
Serum Folate (ng/mL), n (%)			
3.5 - 8.4	3 (27.3)	6 (40)	9 (34.6)
8.5 - 13.4	4 (36.3)	8 (53.3)	12 (46.1)
13.5 - 18.4	3 (27.3)	1 (6.7)	4 (15.4)
> 18.5	1 (9.1)	0 (0)	1 (3.9)
Mean ± SD	11.7 ± 6.3	9.2 ± 3.3	10.3 ± 4.9
Smoking Consumption (packs/week), n (%)			
0	11 (100)	8 (53.4)	19 (73.1)
≤ 2	0 (0)	5 (20)	5 (19.2)
3 - 6	0 (0)	2 (13.3)	2 (7.7)
≥ 7	0 (0)	2 (13.3)	2 (7.7)
Alcohol Consumption (cups/week), n (%)			
≤ 7	11 (100)	9 (60)	20 (76.9)
8 - 28	0 (0)	6 (40.0)	6 (23.1)
> 28	0 (0)	0 (0)	0 (0)

Table 3. Methanol and Formate Concentrations in Serum and Urine

Test	Time	n	Exposed Mean ± SD	Control Mean ± SD	Exposed-Control Mean ± SD	t	p
Serum Formate (mg/L)							
	0 hr	26	11.2 ± 8.1	10.3 ± 5.5	0.9 ± 6	0.76	0.45
	1 hr	25	10.9 ± 5.4	11.7 ± 6.5	-0.6 ± 3.8	-0.77	0.45
	4 hr	26	14.3 ± 8.9	12.7 ± 6.4	1.6 ± 9.2	0.87	0.39
	8 hr	21	12.5 ± 6.3	10.8 ± 5.9	0.5 ± 4.2	0.51	0.61
AUC (mg*hr/L)	0-8 hr	26	101 ± 50	98 ± 43	3.4 ± 34	0.51	0.62
Urine Formate (mg/4 hr)							
	0-4 hr	25	2.2 ± 1.7	1.7 ± 1	0.5 ± 1.4	1.80	0.09
	4-8 hr	25	1.0 ± 0.7	1.0 ± 1.1	0 ± 1.1	0.17	0.87
Serum Methanol (mg/L)							
	0 hr	21	1.4 ± 1.1	1.0 ± 0.6	0.4 ± 1.2	1.34	0.20
	1 hr	21	3.9 ± 2	0.9 ± 0.6	3.1 ± 1.8	7.42	0.00
	4 hr	22	6.1 ± 1.5	0.9 ± 0.6	5.1 ± 1.5	15.94	0.00
	8 hr	22	3.2 ± 2.5	1.8 ± 1.6	1.4 ± 2.6	2.61	0.02
AUC (mg*hr/L)	0-8 hr	22	35.9±12.6	9.3 ± 4.7	26.6 ± 11.7	10.7	0.00
Urine Methanol (mg/4 hr)							
	0-4 hr	26	0.9 ± 0.7	0.2 ± 0.6	0.8 ± 1	4.04	0.00
	4-8 hr	26	0.4 ± 0.2	0.1 ± 0.1	0.3 ± 0.3	5.88	0.00

TB3 JO/2JOST

Table 4. Absorption and Elimination Curve Fits

	Adjusted r^2 Fits			
	Absorption		Elimination	
Subject	Log	Linear*	Log	Linear
1	0.24152	-0.19995	-0.90247	-0.83746
2	0.68485	0.86502	0.98179	0.96152
3	0.61375	0.76214	0.21022	0.26814
4	0.62475	0.74215	-0.16867	-0.08040
5	0.70946	0.61103	0.35912	0.34044
6	0.71127	0.63900	0.45171	0.21840
7	0.76435	0.83332	0.66634	0.57842
8	0.77249	0.77385	0.96442	0.97698
9	0.78816	0.93108	0.48563	0.52921
10	0.60777	0.73996	0.96715	0.89460
11	0.56922	0.61646	0.94377	0.99940
12	0.74480	0.76390	0.75930	0.75333
13	-0.10493	-0.15961	0.59064	0.60410
14	0.82519	0.97914	0.79445	0.97142
15	0.84580	0.98907	0.96161	0.90130
16	0.61160	0.88580	0.96375	0.95318
17	0.78425	0.89271	0.96494	0.91512
18	0.76037	0.83074	0.94596	0.86143
19	0.59171	0.58102	0.99510	0.94388

* Biphasic 0-0.75 hr and 1-3.5 hr; fits only for second phase.

Table 5. Methanol Kinetics Parameters

		Exposed (n = 19)	
Parameter	Units	Mean	SD
SM_0	mg/L	1.81 (0.97)*	2.80 (0.59)
SM_j	mg/L	1.40	1.46
SM_4	mg/L	6.37 (0.79)	2.89 (0.48)
SM_8	mg/L	2.39 (1.30)	1.30 (1.47)
K_a	hr^{-1}	0.91	0.59
K_e	hr^{-1}	-0.26	0.12
$t_{1/2}$	hr	2.62	1.17
T_i	hrs	13.24	6.37
AUC_E	mg·hr/L	31.32	15.52
V_{Dp}	L	38.96	6.05
V_{Dest}	L	63.90	48.56
Cl_t	L/hr	13.46	9.34
Cl_t/wt	L/hr/kg	0.18	0.14
Cl_R	L/hr	0.04	0.02

* Values in parentheses are corresponding concentrations for control condition.
SM = serum methanol at time indicated, SM_j = initial increase in SM (biphasic), V_{Dp} = V_D predicted based on weight x 0.6, V_{Dest} = V_D based on dose/($AUC_E \cdot K_e$), $t_{1/2}$ = half life.

Table 6. Kinetic Formulas

K_a = absorption rate constant, slope of fitted linear absorption

K_e = elimination rate constant, slope of fitted logrithmic elimination curve

K_s = logrithmic absorption rate constant corrected for logrithmic elimination (K_a - K_e)

AUC_E = area under the methanol time curve with endogenous methanol (control) area subtracted and elimination extrapolated to the interception of endogenous levels

T_I = predicted (based on Ke) time to return to control mean methanol concentrations

dose = 250 mg/1000L · MV · 360 minutes · 58% retention

Cl_t = total clearance as estimated by dose/AUC_E

Cl_r = renal clearance as estimated by (urine amounts (mg) for 8 hours)/(serum AUC_8)

V_{Dest} = estimated volume of distribution = dose/AUC_E·Ke)

MV = minute ventilation
for females MV = 0.212 BPM + 5.599 BSA - 1.941*
for males MV = 0.212 BPM + 5.086 BSA - 3.40*
* CARB A033-205, 1993

BPM = breaths per minute

BSA = body surface area in cm^2 = hlt(cm) $^{0.725}$ + Wt(kg) $^{0.425}$ x 71.84

Table 7. Neurobehavioral and Visual Test Scores

Test	Control				Exposed			
	Pre-Exposure		Post-Exposure		Pre-Exposure		Post-Exposure	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
P300 Amplitude	7.87	4.20	7.20	2.84	7.88	2.99	7.00*‡	2.80
P300 Latency	326.26	25.90	331.20	21.32	328.96	23.59	327.30	20.17
2 & 7	317.15	54.11	316.15	52.03	314.19	52.09	316.04	48.36
Symbol Digit	58.00	10.40	63.50*	8.05	59.62	13.64	62.85*‡	10.83
CCI (Lanthony)	1.22	0.26	1.21	0.29	1.17	0.26	1.17	0.23
Vistec 1.5 cpd	70.38	13.75	76.99*	15.94	72.82	14.65	71.99†‡	14.16
Vistec 3 cpd	92.63	15.12	93.83	18.99	99.73	24.20	96.99	19.91
Vistec 6 cpd	125.96	21.48	128.27	13.26	128.40	18.54	131.09	19.80
Vistec 12 cpd	84.67	15.88	87.85	13.93	84.96	18.00	90.33*	12.87
Vistec 18 cpd	29.50	8.37	32.05*	8.27	29.18	8.57	34.26*	9.10
Sternberg/Slope	36.53	41.81	52.36	44.71	38.22	29.84	37.71	32.50
Intercept	676.72	185.78	624.34	148.41	640.49	114.59	663.79	138.42
Stroop/Formula	-0.22	7.68	3.32*	9.97	-0.28	6.30	2.48*	6.45
Color Word	45.65	10.28	49.85*	12.34	45.08	8.51	48.85*	8.88

* Significant ($p < 0.05$) within condition change (pre vs. post) by t-test
† Significant ($p < 0.05$) between condition difference in scores (pre vs pre or post vs post) by t-test
‡ Significant ($p < 0.05$) between condition difference for changes in scores by t-test
· Significant ($p < 0.05$) between condition difference for changes in scores by MANOVA

2TABLE7 JO/2JOST

Table 8. Comparison of NBT and Visual Tests Score by Differences

Test	Variable	Score	Exposed-Control Difference in Score						Pre-Post Chamber Change in Score						Differences of the Changes *		
		Baseline Mean ± SD	Pre Chamber Mean ± SD	t	p	Post Chamber Mean ± SD	t	p	Exposed difference Mean ± SD	t	p	Control difference Mean ± SD	t	p	Exp. - Con. Mean ± SD	t	p
Symbol Digit		62.1 ± 11.8	2.6 ± 8.7	1.50	0.15	1.0 ± 8.0	-0.63	0.54	3.1 ± 10.5	53*	0.08*	6.7 ± 7.3	-4.58	0.00	-3.6 ± 9.2	-2.0	0.06
Stroop	Formula	-1.6 ± 5.2	0.1 ± 5.1	0.06	0.95	-0.8 ± 9.2	-0.47	0.64	2.8 ± 6.1	2.30	0.03	3.5 ± 7.5	2.40	0.02	-0.8 ± 10.8	22.5*	0.58*
	Color Word	38.2 ± 6.3	-0.6 ± 5.7	0.51	0.61	-1.0 ± 8.7	-0.59	0.56	3.8 ± 5.6	3.45	0.00	4.2 ± 6.6	3.26	0.00	-0.4 ± 9.8	18.5*	0.61*
Sternberg	Intercept	676.7 ± 185.8	-36.2 ± 208.3	-0.89	0.38	39.4 ± 160.9	1.25	0.22	23.3 ± 154.7	0.77	0.45	-52.4 ± 188.1	-1.42	0.17	75.7 ± 237.3	1.63	0.12
	Slope	48.2 ± 41.6	1.7 ± 48.1	0.18	0.86	11.7 ± 38.9	1.92	0.07	0.5 ± 38.8	-0.07	0.95	15.8 ± 54.5	1.48	0.15	-16.4 ± 63.5	-1.31	0.21
2 & 7		316.2 ± 56.2	-3.0 ± 29.5	0.51	0.61	-0.1 ± 30.6	0.02	0.98	1.9 ± 26.7	0.35	0.73	1.0 ± 21.8	0.23	0.82	2.9 ± 29.9	0.49	0.63
Vistech	1.5 cpd	68.2 ± 14.7	2.4 ± 13.1	0.88	0.39	-5.0 ± 9.4	2.71	0.01	-0.8 ± 13.3	-0.32	0.75	6.6 ± 14.5	2.33	0.03	-7.4 ± 16.6	-36*	0.04*
	3.0 cpd	92.7 ± 13.2	4.0 ± 21.2	0.95	0.35	3.2 ± 11.5	1.40	0.17	2.7 ± 24.1	-9*	0.57*	1.2 ± 19.2	0.32	0.75	7.1 ± 20.1	13.5*	0.13*
	6.0 cpd	123.9 ± 17.9	2.4 ± 12.6	0.93	0.34	2.8 ± 16.3	0.88	0.39	2.7 ± 19.0	0.72	0.48	2.3 ± 19.5	0.60	0.55	0.4 ± 21.0	0.09	0.93
	12.0 cpd	84.2 ± 10.6	0.3 ± 10.4	0.14	0.89	2 ± 11.0	1.15	0.26	5.4 ± 11.7	35*	0.01*	3.2 ± 12.3	1.32	0.20	2.2 ± 16.6	0.67	0.51
	18.0 cpd	29.0 ± 6.4	0.3 ± 6.1	0.27	0.79	2.2 ± 9.4	1.20	0.24	5.1 ± 7.5	3.43	0.00	2.6 ± 5.4	2.40	0.02	2.5 ± 8.7	34.5*	0.24*
Lanthony	CCT	1.3 ± 0.3	0.1 ± 0.2	1.18	0.13	0.0 ± 0.2	0.93	0.35	0.0 ± 0.1	-0.28	0.78	-0.0 ± 0.2	-0.44	0.66	0.0 ± 0.2	0.22	0.83
P300	Amplitude	7.0 ± 2.3	0.3 ± 3.0	0.42	0.68	0.4 ± 1.8	0.93	0.36	1.3 ± 2.0	3.05	0.01	-0.4 ± 2.8	-0.67	0.51	-1.1 ± 3.5	-1.39	0.18
	Latency	322.7 ± 20.8	1.9 ± 18.7	0.38	0.71	-3.2 ± 15.0	1.30	0.21	1.4 ± 16.3	1.25	0.22	2.9 ± 17.3	0.80	0.43	-7.1 ± 22.0	-1.40	0.18

Note:
* Difference (exposed - control) of the changes (post - pre) in test scores
• Signed Rank statistics

Table 9. Methanol and Formate in Serum and Urine in the Folate-Susceptibles

Test	Time	Difference (exposed - control) in the Changes (post - pre) in Concentrations		Concentrations During Methanol Exposure		
		Normal Subjects (mean ± SD)	4 IBD Subjects 802; 803; 806; 903 (individual results)	Normal Subjects (mean ± SD)	2 MTX Subjects Off MTX (807;907 scores)	2 MTX Subjects On MTX (807;907 scores)
Serum	0 hour	0.9 ± 6	ϕ; -6.8; 5.4; 1.8	11.2 ± 8.1	4.5; 13	9.7; 10.6
Formate	1 hour	-0.6 ± 3.8	ϕ; 0; -7.7; -0.7	10.9 ± 5.4	10.2; 19.9	11.8; 22.1
(mg/L)	4 hours	1.6 ± 9.2	ϕ; 3.2; -12.3; -20.6	14.3 ± 8.9	15.4; 26.1	16.9; 2.5
	8 hours	-0.2 ± 4.3	ϕ; -7.1; ϕ; 16.1	12.5 ± 7.3	ϕ; 7.8	12.8; 29.5
Urine Formate	0-4 hours	0.5 ± 1.4	-0.6; 0.1; 13; -2.6	2.2 ± 1.7	5.6; 3.1	4.6; 12.4
(mg/4 hr)	4-8 hours	0 ± 1.1	-0.9; ϕ; -1.7; -1.7	1.0 ± 0.7	2.2; 2.5	1.7; 1.9
Serum	0 hour	0.4 ± 1.2	ϕ; -0.4; 0.1; 0.2	1.4 ± 1.1	0.3; 0.1	1.0; 03
Methanol	1 hour	3.1 ± 1.8	ϕ; 2.5; ϕ; 3	3.9 ± 2	2.2; 2.7	2.9; 2.9
(mg/L)	4 hours	5.1 ± 1.5	ϕ; ϕ; 6.7; 5.3	6.1 ± 1.5	4.7; 4.3	7.3; 6.0
	8 hours	1.8 ± 2.7	ϕ; -0.1; ϕ; 1.3	3.2 ± 2.6	2.0; 2.5	2.3; 1.8
Urine Methanol	0-4 hours	0.8 ± 1	0.2; 0.2; -0.3; 0.4	0.9 ± 0.7	ϕ; 0.2	ϕ; 0.7
(mg/4 hr)	4-8 hours	0.3 ± 0.3	0.2; ϕ; 0.2; ϕ	0.4 ± 0.2	ϕ; 0	ϕ; 0.1

ϕ = missing value

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Table 10. Neurobehavioral Test Results in the Folate-Susceptibles

Test	Difference (exposed - control) in the Changes (post-pre) in Scores		Changes in Scores During Methanol Exposure		
	Normal Subjects (mean ± SD)	4 IBD Subjects 802; 803; 806; 903 (individual results)	Normal Subjects (mean Score ± SD)	2 MTX Subjects Off MTX 807; 907 (individual results)	2 MTX Subjects On MTX 807; 907 (individual results)
P-300 Amplitude	-1.1 ± 3.5	-4; 5; 5; 0	-1.3 ± 2	1; ϕ	-3; 4
P-300 Latency	-7.1 ± 22	0; -32; -32; 4	-4.4 ± 16.3	0; ϕ	0; 0
Symbol Digit	-3.6 ± 9.2	-2; 0; 3; 6	3.1 ± 10.5	8; -2	11; 1
2-7	2.9 ± 29.9	2; -50; 35; 36	1.9 ± 26.7	-17; 1	-14; 3
Lanthony (CCI)	0 ± 0.2	0; -0.1; 0.2; 0.1	0 ± 0.1	-0.1; -0.1	0; 0-5
Stroop (Formula)	-0.8 ± 10.8	-0.4; -5.7; -4.1; -0.3	2.8 ± 6.1	1.1; -5.8	3.8; -2
Stroop (Color-Word)	-0.4 ± 9.8	-3; -5; -3;1	3.8 ± 5.6	1; -4	6; -4
Vistech 1.5	-7.4 ± 16.6	-40; -23.3; -61.7; 0	-0.8 ± 13.3	-5; -11.7	11.7; 0
Vistech 3	7.1 ± 20.1	28.3; 0; 28.3; 0	-2.7 ± 24.1	0; 0	0; 13.7
Vistech 6	0.4 ± 21	-15; 0; 0; 0	2.7 ± 19	8.3; -36.7	0; -26.7
Vistech 12	2.2 ± 16.6	-5.3; 7.7; -37; 12.3	5.4 ± 11.7	40; 35.3	-2; 0
Vistech 18	2.5 ± 8.7	-10.7; 2.7; 16.7; 13	5.1 ± 7.5	2.7; 6.3	-7.2; -4.7
Sternberg Slope	-16.4 ± 63.5	-55.5; 4.8; -54.8; 35.8	-0.5 ± 38.8	30.8; -25.7	9.7; 63.8
Sternberg Intercept	75.7 ± 237.3	151.4; -54.3; 408.1; -163	23.3 ± 154.7	-106; 70.5	-91.3; 312

TB10 JP/2JOST

Table 11 Selected Correlations Between Predictor Covariates and Outcome Variables

	P3amp dif.	P3lat dif	P3lat post	SyD post	2.7 dif.	2.7 post	CCI dif	Stroop post	CW dif	Vis1 dif	Vis1 post	Vis3 dif	Vis3 post	Vis6 dif	Vis12 dif	Vis12 post	Vis18 dif	Vis18 post	Ster dif	Ster. post	Ster. Int dif	Ster Int po
	-0.43																-0.50	-0.43	-0.42	-0.53		0.49
SM4	0.09																0.02	0.05	0.05	0.01		0.02
	16																22	22	22	22		22
														0.43	-0.52				0.55	-0.50	0.54	0.48
UM4														0.03	0.01				0.00	0.01	0.00	0.01
														26	26				26	26	26	26
SM	0.48										0.47									-0.50		0.49
auc4	0.06										0.03									0.02		0.02
	16										22									22		22
SM	-0.46										-0.59									-0.57		-0.56
auc6	0.07										0.03									0.01		0.01
	16										22									22		22
SM	-0.45										-0.42									-0.47		-0.50
auc8	0.08										0.04									0.03		0.02
	16										22									22		22
		-0.56		0.47				-0.39	-0.34				-0.52				-0.43					
Folate		0.01		0.07				0.05	0.10				0.01				0.03					
		19		25				25	25				25				25					
													0.334							0.49		-0.53
Gender													0.10							0.01		0.01
													25							25		25
			0.38	-0.37	0.34	0.37	0.35															
Age			0.09	0.07	0.10	0.07	0.08															
			21	25	25	25	26															
				0.53		0.50				0.39					0.42	0.39	0.37					
Order				0.01		0.01				0.05					0.04	0.05	0.07					
				25		26				25					25	25	25					
	0.66																	0.34				
Alcohol	0.00																	0.09				
	19																	26				
												0.47					0.35					
Smoking												0.08					0.08					
												26					26					

dif = difference (exposed - control) of the changes (post - pre exposure) in the factor; post = difference in post exposure test scores (exposed - control); P3amp = P300 amplitude

P3lat = P300 latency, SyD = Symbol Digit, CCI = Cauthorn, Vis = Vistech, Ster = Sternberg, Int = Intercept

Figure 1

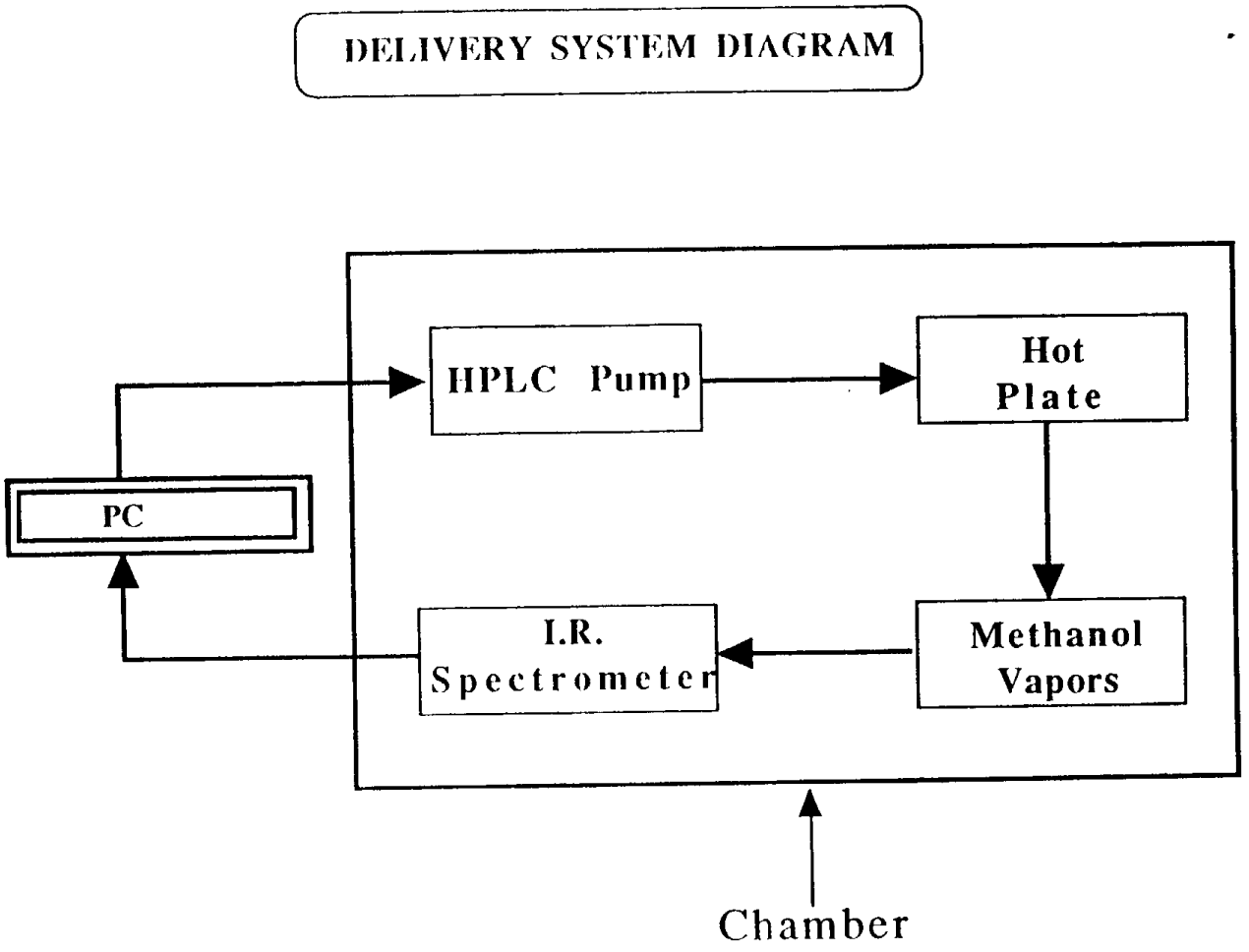

DELIVERY SYSTEM DIAGRAM
HPLC Pump
Hot Plate
PC
I.R. Spectrometer
Methanol Vapors
Chamber

Figure 2

Methanol Concentration in the Chamber

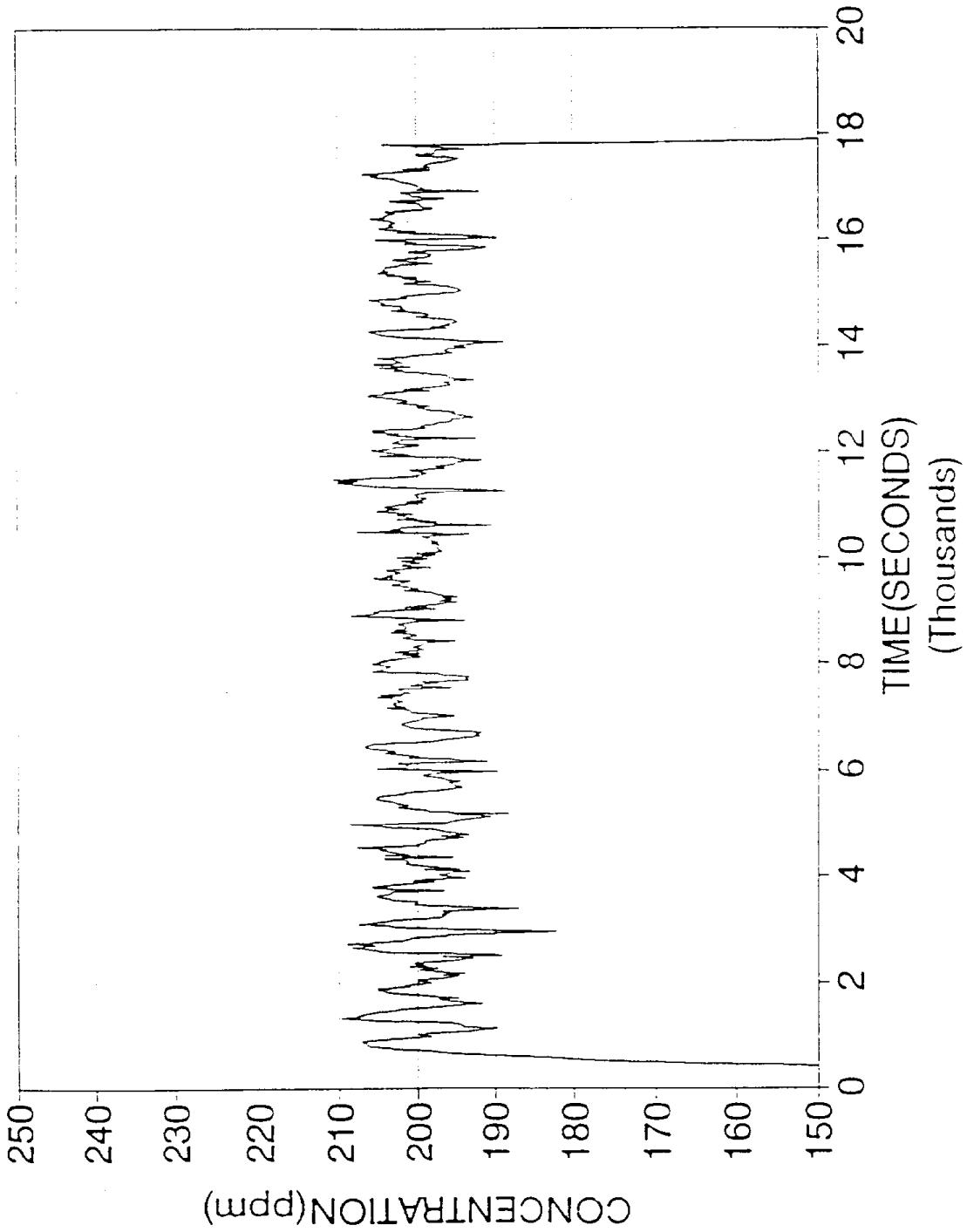
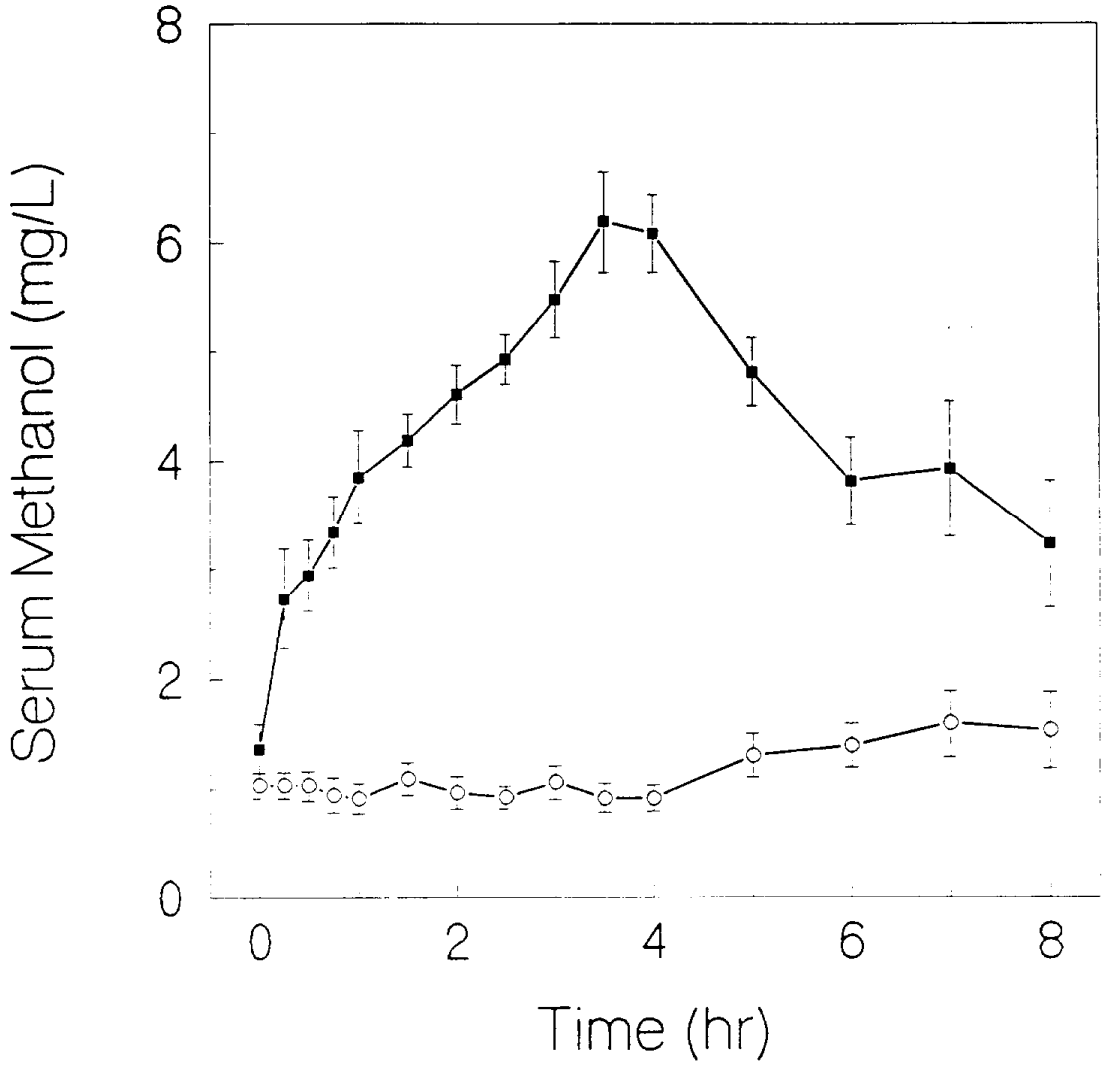
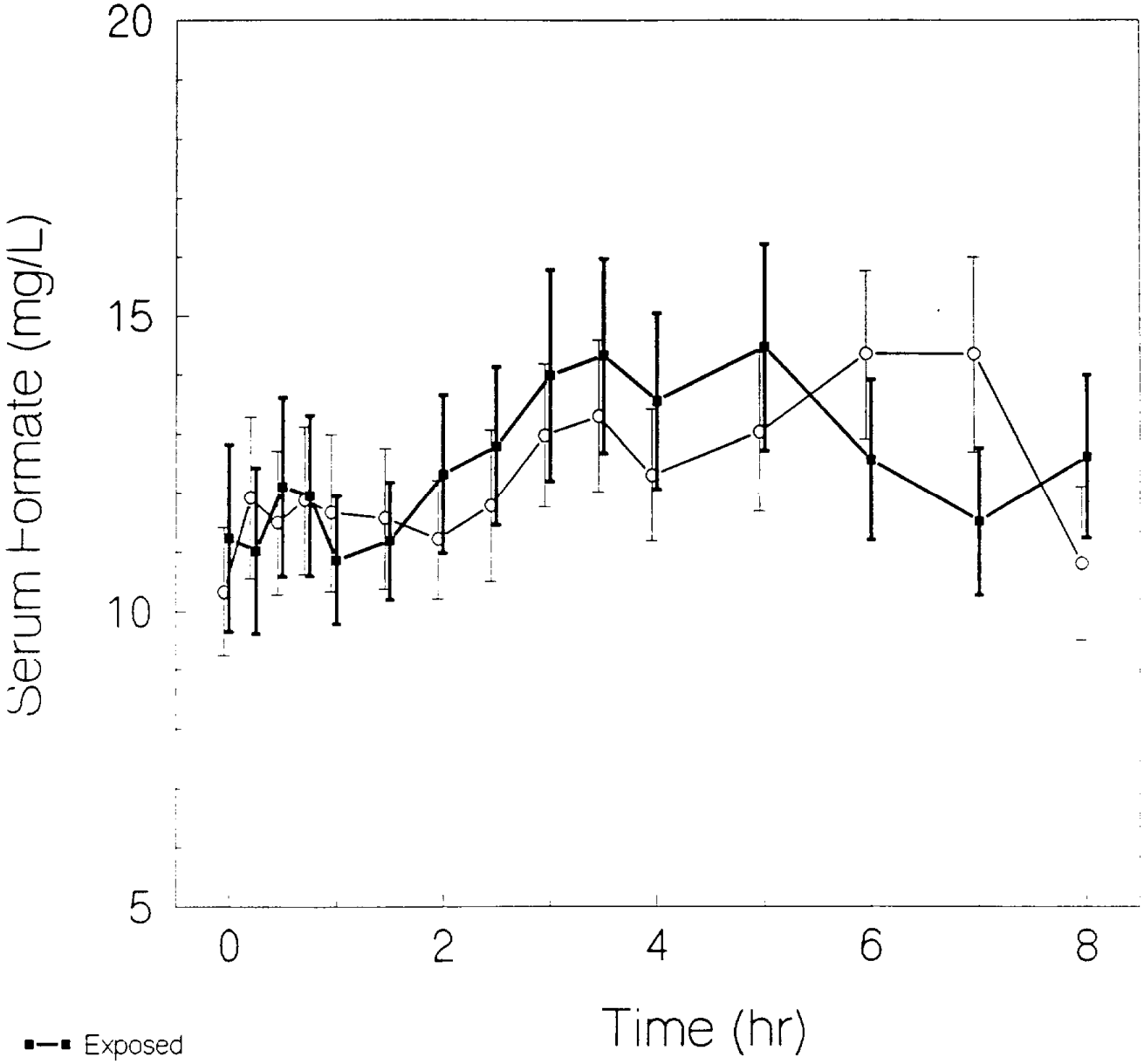


Figure 3
Serum Methanol Concentration Over Time



Error bars are +/- one standard error.

Figure 4
Serum Formate Concentration Over Time



Error bars are +/- one standard error.

Appendix A

Neurobehavioral tests in Other Studies - Consistency, Sensitivity, and Specificity

Author/year	Subjects	NBT	Exposure	Consistency Sensitivity Specificity of NBT	Dysfunctions found	Limitations	Strength
Trimble & Thompson, 1983.	Healthy volunteers + patients with epilepsy.	Stroop, 2 & 7, and other tests.	Anticonvulsant drugs: phenytoin, carbamazepine, sodium valporate, dobozam, and placebo (only for the healthy volunteers).	Specificity of NBT to discriminate patient group from control.	Cognitive functions: memory; attention; retention of new inform.; speed and accuracy of perceptual registration; manual speed; decision making.	Small samples in each group.	Dose response effects, substitution therapy effect, serum level effects.
Greenberg et al., 1985.	12 male, age 18-36.	Symbol Digit and other 8 NES tests.	* 20% N_2O + 80% Oxygen * Control: 100% Oxygen	N_2O impaired performance of Symbol Digit and other two tests.	Psychomotor performance	Small sample size.	Use of new neurobehavioral evaluation test (NES).
Mahoney et al., 1988.	15 male, age 24-34.	Symbol Digit and other tests.	* 40% N_2O * Control * 20% N_2O.	Impaired performance on Symbol Digit at 20 & 40% N_2O Significant "practice" effect in Symbol Digit.	Psychomotor performance	One tailed t - test (high risk of type I error).	Dose response relationship, reproducibility of other studies.
Hoolsma et al., 1988.	10 male, age 26-44	Control word vigilance tests (similar to Stroop) and others tests.	35% ethanol or control.	Color word vigilant test was affected at alcohol blood levels of 0.03%.	Processing verbal information, switching of attention, degree of complex cognitive processing.	* Small number of subjects * Moderate users of alcohol (received different amounts of alcohol at different times).	Acute and one level of alcohol exposure.

Appendix A

(continues)

Author-year	Subjects	NBT tests	Exposure	Consistency Sensitivity Specificity of NBT	Dysfunctions found	Limitations	Strength
Letz et al., 1990.	118 workers at five fiberglass boat building companies.	Symbol digit and other two NES tests.	Styrene exposure assessment at workplace.	Symbol Digit test significantly related to styrene exposure while adjusted age & education. High test-retest correlation.	Psychomotor performance	Industries were not randomly selected.	* Exposure measurement * Biological monitoring (urinary mandelic acid).
Cook et al. 1991.	12 healthy young male.	Symbol digit, Stroop, Sternberg, and other tests.	* Methanol (192 ppm) * Sham	Decrement in Sternberg.	Fatigue, less vigor and concentration (small effects).	Small homogenous sample size. One level of exposure.	Good recommendations for future studies.
Hanninen et al., 1991.	10 female and 11 male, age 28-55, monozygotic twins + control of 28 pair twins.	Stroop, Symbol digit and other tests.	Solvent mixtures 5-30 years. Higher exposure in previous years.	Stroop: slower in the cognitive interference task.	Verbal learning, memory, & cognitive functions for visuoconstructive task.	Small sample size. Exposures range from low to moderate.	Monozygotic cotwins as controls for the exposed subjects.
Dick et al., 1992.	68 males - 75 females (18-32) randomly assigned to one of six treatment groups.	Sternberg and other tests.	Control, MEK, MIBK, MEK & MIBK (vapors) + ethanol, alcohol placebo (ingestion).	Sternberg: no effect in alcohol exposure. Decrement in other NBT (on at least one measure of each). Gender difference no related to higher blood alcohol level	No significant neurobehavioral effects.	Young population (18-32).	Combination and control of different exposures.
Kishi et al., 1993.	20 painters for NBT + matched reference.	Digit Symbol and other tests.	Chronic low level exposure to mixed solvents (included methanol).	Digit Symbol significantly lower in exposed than control.	Psychomotor performance	No matching in alcohol consumption (exposure > control). Small sample size.	Symptom questionnaire added to NBT.

Appendix B

Study Advertising

CENTER FOR OCCUPATIONAL AND ENVIRONMENTAL HEALTH

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

The Center for Occupational and Environmental Health (COEH) of the University of California is seeking men and women between the ages of 20 and 51 to participate in a chamber study with exposure to low levels of methanol at UCSF. Blood and urine samples will be taken during the exposure as well as special eye and neurological tests. Two full days of participation are necessary, and one half day of training. An honorarium of $500 is provided for completion of the full study. If you are interested call for more information at 415-206-5200 and ask for Charles E. Becker, MD, or Patricia Chuwers, RN, MPH.

Appendix c

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO.

CONSENT TO BE A RESEARCH SUBJECT

PROJECT TITLE:

Neurological Effects of Low Level Methanol in Normal Humans

PURPOSE AND BACKGROUND:

Charles Becker, MD, Patricia Chuwers (RN, MPH), and their colleagues are conducting a study to learn whether the new automotive fuel, methanol, poses any risk to the eye and nervous system of healthy human subjects with documented normal amount of the vitamin folic acid. Because I am a healthy person, I am being asked to participate in this study.

PROCEDURE:

If I agree to be in this study, the following will happen:

1. I will meet with Dr. Becker or his associates and provide to them my medical history, and allow her to do a physical examination and to draw a sample of 10cc (1/3 of an ounce) blood to ensure that I have a sufficient amount of the vitamin folic acid in my blood.

2. I will agree to being on a diet free of alcohol, fruit juice, vegetables, roast coffee and aspartame (NutraSweet brand sweetener) for one day prior to my two studies.

3. I will agree to spend four hours in a 8 x 8 x 8 exposure chamber at the University of California, San Francisco on two occasions at least three weeks apart. In the chamber, methanol will be circulated in the air at a concentration of 250 mg/m^3 at the two occasions. This amount of methanol exposure is below amounts accepted for workers 8 hours per

day, 5 days a week for working life. On the other occasion, room air will be circulated in the chamber. I will not be told which occassion is the methanol exposure.

4. A small needle will be placed in my arm which will allow for blood sampling (5 cc's, about 1/6 of an ounce of blood) once before I enter the chamber, every 15 minutes for the first hour, and then every 30 minutes for 1-4 hours, and then every 60 minutes for 4 hours after I leave the exposure chamber.

5. Before, during and after the time of my being in the exposure chamber, I will perform a special series of tests involving listening to special tones through earphones, color vision testing, and counting numbers and letters. I will be trained for 1 hour how to perform the tests before the start of the study.

6. I will pass my urine before entering the exposure chamber and collect all my urine during the four hours of exposure and for an additional four hours after exposure stops.

RISK AND DISCOMFORT:

1. Placing an intravenous heparin-lock in my arm may cause some bruising and discomfort and a remote possibility of a small local infection. The risk of placing this needle in my arm is a temporary discomfort from a needle stick and bruising.

2. I will remain in an exposure chamber for four hours, and may become bored or frightened being in a chamber. I can see through the glass partition in the chamber and tell Patricia Chuwers, RN, MPH how am I feeling. During the exposure I'm going to share the chamber with another one or two volunteers.

3. For four hours in the exposure chamber I will be exposed to methanol. I do not anticipate that I will have any symptoms from this exposure. It is possible that I may have a slight headache or eye irritation.

CONFIDENTIALITY:

Participation in this research project may involve some loss of privacy. My research records will be handled as confidentially as possible within the law. All of my records will be coded and kept in a locked file so that only Dr. Becker and Patricia Chuwers, RN, MPH will have access to them. No individual identities will be used in any of the reports or publications as a result of this study. Only Dr. Becker and Patricia Chuwers, RN, MPH will know the results of my tests.

TREATMENT AND COMPENSATION FOR INJURY:

If I am injured as a result of being in this study, treatment will be available. The cost of such treatments may be covered by the University of California depending on a number of factors. The University does not normally provide any other form of compensation for injury. For further information about this I may call the office of the Committee of Human Research at (415) 476-1814, or write the Committee on Human Research at Box 0962, UCSF, San Francisco, CA 94143.

BENEFITS:

There will be no direct benefits to me from participating in this study. However, it is hoped that the information gained from this study will allow Dr. Becker and his colleagues to predict more accurately how the alternative fuel, methanol, will be handled in healthy subjects with documented normal folic acid, and to anticipate any visual or neurological effects that might occur at these low levels.

CHARGES:

I will not be charged for any of the study procedures in this project.

REIMBURSEMENT:

I will be reimbursed $500 by check within two weeks for the three meetings, that means full participation. If I decide to withdraw prior to the study completion, or if Dr. Becker or his colleagues decides to terminate my study participation, I will receive $50 by check within two weeks. If I complete only one training meeting and one exposure session I will recieve $250 by check within two weeks.

QUESTIONS:

I have talked with Dr. Becker or his colleagues about this study and have had my questions answered. If I have any further questions about this study I may call (415) 206-5200.

CONSENT:

I have been given a copy of this consent form and the Experimental Subjects' Bill of Right to keep. Participation in this research is voluntary. I am free to decline to be in this study or withdraw from it at any time.

_____	_____
Subject's Signature	Date
_____	_____
Person Obtaining Consent	CHR Number

Appendix D

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO.

CONSENT TO BE A RESEARCH SUBJECT

PROJECT TITLE:

Neurological Effects of Low Level Methanol in Folate-Deficient Humans

PURPOSE AND BACKGROUND:

Dr John Osterloh, Patricia Chuwers (RN, MPH), Dr. D'Alessandro and their colleagues are conducting a study to learn whether exposure to a possible automotive fuel ingredient methanol, poses any risk to the eye and nervous system of subjects with documented low to normal vitamin folic acid stores. Because I am in a susceptible group that may have low levels of vitamin folic acid, I am being asked to participate in this study.

PROCEDURE:

If I agree to be in this study, the following will happen:

1. I will meet with Dr. D'Alessandro and provide her my medical history, and allow her to do a physical examination and to draw a sample of 10cc (1/3 of an ounce) blood to asses the vitamin folic acid level in my blood.

2. I will agree to consume a diet free of alcohol, fruit juice, vegetables, roast coffee and aspartame (NutraSweet brand sweetener) for one day prior to my two studies.

3. I will agree to stop for a day the medications that can interfere with the results of the tests (Vitamin C, Azulfidine, Methotrexate).

4. I will agree to spend four hours in a 8 x 8 x 8 exposure chamber at the University of California, San Francisco on two occasions at least three weeks apart. In the chamber,

methanol will be circulated in the air at a concentration of 250 mg/m^3 at the two occasions. This amount of methanol exposure is below amounts accepted for workers 8 hours per day, 5 days a week for working life.

5. A small needle will be placed in my arm which will allow for blood sampling (5 cc's, about 1/6 of an ounce of blood) once before I enter the chamber, every 15 minutes for the first hour, and then every 30 minutes for 3 hours, and then every 60 minutes for 4 hours after I leave the exposure chamber.

6. Before, during and after the time of my being in the exposure chamber, I will perform a special series of tests involving listening to special tones through earphones, color vision testing, and counting numbers and letters. I will be trained for 1 hour to how to perform the tests in the first meeting.

7. I will pass my urine before entering the exposure chamber and collect all my urine during the four hours of exposure and for an additional four hours after exposure stops.

8. Only at the end of the first day of exposure I will get folic acid replacement.

RISK AND DISCOMFORT:

1. Placing an intravenous heparin-lock in my arm may cause some bruising and discomfort and a remote possibility of a small local infection. The risk of placing this needle in my arm is a temporary discomfort from a needle stick and bruising.

2. I will remain in an exposure chamber for four hours, and may become bored or frightened being in a chamber. I can see through the glass partition in the chamber and tell Patricia Chuwers, RN, MPH how am I feeling. During the exposure I'm going to share the chamber with another one or two volunteers.

3. For four hours in the exposure chamber I will be exposed to methanol. I do not anticipate that I will have any symptoms from this exposure. It is possible that I may have a

slight headache or eye irritation.

CONFIDENTIALITY:

Participation in this research project may involve some loss of privacy. My research records will be handled as confidentially as possible within the law. All of my records will be coded and kept in a locked file so that only Dr. Osterloh, Dr D'Alessandro and Patricia Chuwers will have access to them. No individual identities will be used in any of the reports or publications as a result of this study. Only Dr. Osterloh, Dr. D'Alessandro and Patricia Chuwers will know the results of my tests.

TREATMENT AND COMPENSATION FOR INJURY:

If I am injured as a result of being in this study, treatment will be available. The cost of such treatments may be covered by the University of California depending on a number of factors. The University does not normally provide any other form of compensation for injury. For further information about this I may call the office of the Committee of Human Research at (415) 476-1814, or write the Committee on Human Research at Box 0962, UCSF, San Francisco, CA 94143.

BENEFITS:

There will be no direct benefits to me from participating in this study. However, it is hoped that the information gained from this study will allow Dr. Osterloh and his colleagues to predict more accurately how the alternative fuel, methanol, will be handled in folate deficient subjects, and to anticipate any visual or neurological effects that might occur at these low levels.

CHARGES:

I will not be charged for any of the study procedures in this project.

REIMBURSEMENT:

I will be reimbursed $500 by check for the three meetings, that means full participation. If I decide to withdraw prior to the study completion, or if Dr. Osterloh decides to terminate my study participation, I will receive $50 by check within two weeks.

QUESTIONS:

I have talked with Dr. Osterloh or his colleagues about this study and have had my questions answered. If I have any further questions about this study I may call Patricia Chalwers by (415) 564-[illegible]

CONSENT:

I have been given a copy of this consent form and the Experimental Subjects Bill of Right to keep. Participation in this research is voluntary. I am free to decline to be in this study or withdraw from it at any time.

_____	_____
Subject's Signature	Date
_____	_____
Person Obtaining Consent	CHR Number

Appendix E

Dear,

Dr. Maibach, Dr. John Osterloh, Patricia Chuwers, RN, MPH, and their colleagues from the University of California, San Francisco are conducting a study to learn whether exposure to a possible automotive fuel ingredient methanol, that is expected to improve air quality, relative to regulated pollutants, poses any risk to the eye and nervous system of subjects with methotrexate treatment.

The current study has been designed in order to evaluate, in a controlled condition, the effect of 4 hours exposure to low gaseous dose of methanol (200ppm).

In the first phase of the project we studied 26 healthy volunteers and we found no differences between the exposure and the control condition. In the second phase we are studying subjects that are affected by psoriasis and are treated with low doses of drug methotrexate.

The study consist of three sessions. The first is a meeting, lasting about two hours, in which volunteers are requested to fill in a questionnaire and to give their consent to participate in the study. Then a physician will collect medical history and perform a physical examination. Neurobehavioral tests are also administered. The second and third sessions are lasting nine hours each and held on weekends. Volunteers are requested to stay for four hours in the exposure chamber each day. Blood and urine are collected at regular time intervals for eight hours. Neurological and visual tests are administered before volunteers enter the chamber and again after the exposure.

In the first meeting we'll explain all the details of the research, and you have then, another opportunity to decide if you are willing to participate or not.

You will reimbursed $500 by check for the three meetings, that means full participation.

Please, let us know your answer by calling Patricia Chuwers at the telephone number 864-1604.

We'll really appreciate your first agreement

Sincerely,

Patricia Chuwers, RN, MPH

Appendix F

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

EXPERIMENTAL SUBJECT'S BILL OF RIGHTS

The rights below are the rights of every person who is asked to be in a research study. As an experimental subject I have the following rights:

1) To be told what the study is trying to find out.

2) To be told what will happen to me and whether any of the procedures, drugs, or devices is different from what would be used in standard practice.

3) To be told about the frequent and/or important risks, side effects or discomforts of the things that will happen to me for research purposes.

4) To be told if I can expect any benefit from participating, and, if so, what the benefit might be.

5) To be told the other choices I have and how they may be better or worse than being in the study.

6) To be allowed to ask any questions concerning the study both before agreeing to be involved and during the course of the study.

7) To be told what sort of medical treatment is available if any complications arise.

8) To refuse to participate at all or to change my mind about participation after the study is started. This decision will not affect my right to receive the care I would receive if I were not in the study.

9) To receive a copy of the signed and dated consent form.

10) To be free of pressure when considering whether I wish to agree to be in the study.

If I have other questions I should ask the researcher or the research assistant. In addition, I may contact the Committee on Human Research, which is concerned with protection of volunteers in research projects. I may reach the committee office by calling: (415) 476-1814 from 8:00 AM to 5:00 PM, Monday to Friday, or by writing to the Committee on Human Research, University of California, San Francisco, CA 94143.

Call X1814 for information on translations.

NOTE: We will provide translations of the English version in Spanish and Chinese languages.

Appendix G

Interview for the Methanol Study

ID Number ___ ___ ___

---

1. When were you born?

___ ___/___ ___/___ ___
mo day yr

2. What gender are you?

[1] Male [2] Female

3. Which category best describes your ethnic background?

[1] Hispanic/Latino [4] Caucasian
[2] Black [5] Asian
[3] American Indian [6] Other _____ ___

4. Please circle the highest year of school that you completed. Circle one bracketed grade only.

Elementary School	[01] [02] [03] [04] [05] [06]
Junior High School	[07] [08] [09]
High School	[10] [11] [12]
College/Trade School	[13] [14] [15] [16]
Graduate School	[17]

5. Do you have any hobbies in which you use chemicals?

[1] Yes [2] No

6. Please list your previous occupations.

a. _____ [___]
b. _____ [___]
c. _____ [___]
d. _____ [___]

7. Did you use any chemicals in any of your previous occupations?

[1] Yes [2] No

8. If you used chemicals in any of your jobs, please explain.

a. _____ [___]
b. _____ [___]
c. _____ [___]

9. Did you wear protective clothing such as gloves or a hood or a respirator in any of your previous occupations?

[1] Yes [2] No

10. What is your present occupation?

_____ [___]

11. Do you use any chemicals in your present occupation?

[1] Yes [2] No

12. If you use chemicals in your present occupation, please explain here.

a. _____ [___]
b. _____ [___]
c. _____ [___]

13. Do you presently work the night shift?

[1] Yes [2] No

Medical History

14. Do you take any medications prescribed by a physician?

[1] Yes [2] No

15. If so, please list them here.

a. _____ ___
b. _____ ___
c. _____ ___
d. _____ ___
e. _____ ___

16. Do you regularly take any over the counter medications not prescribed by a physician?

[1] Yes [2] No

17. If so, please list them here.

a. _____ ___
b. _____ ___
c. _____ ___
d. _____ ___
e. _____ ___

18. Do you have or have you ever had any of the following problems diagnosed by a physician?

	Yes	No	Don't Know
a. Kidney Disease (Not Stones)	[1]	[2]	[9]
b. Gout	[1]	[2]	[9]
c. Psychiatric problems requiring treatment	[1]	[2]	[9]
d. Diabetes Mellitus (High Blood Sugar)	[1]	[2]	[9]
e. Hypertension (High Blood Pressure	[1]	[2]	[9]
f. Heart Disease	[1]	[2]	[9]
g. Alcoholism	[1]	[2]	[9]
h. Liver Problems (hepatitis, etc.)	[1]	[2]	[9]
i. Nervous System Disorders	[1]	[2]	[9]

19. If you have any other medical conditions that you feel might be impotant, please list them here.

a. _____ [___]
b. _____ [___]
c. _____ [___]
d. _____ [___]
e. _____ [___]

20. On average, about how many cups of coffee (with caffeine) do you drink in one week?

[1] Less than 7 cups per week
[2] Between 8 and 28 cups per week.
[3] More than 28 cups per week (4 cups per day or more.

21. Over your lifetime, is this pattern:

[1] Higher than usual
[2] Typical
[3] Lower than usual

22. On average, about how many drinks containing alcohol (including beer and wine) do you drink in one week?

[1] Less than 7 cups per week
[2] Between 8 and 28 cups per week.
[3] More than 28 cups per week (4 cups per day or more.

23. Over your lifetime, is your present drinking pattern:

[1] Higher than usual
[2] Typical
[3] Lower than usual

24. On average, how many packs of cigarettes do you smoke in one week?

[1] I do not smoke
[2] 2 packs or less per week
[3] Between 3 and 6 packs per week
[4] 7 or more packs per week (a pack a day or more)

25. Over your lifetime, is your current smoking pattern:

[1] Higher than usual
[2] Typical
[3] Lower than usual

26. Do you have any allergies?

[1] Yes [2] No

27. If you have allergies, Please explain here.

_____ [___]

28. Have you ever had a blood transfusion?

[1] Yes [2] No

29. Have you ever use a needle to inject drugs into your body?

[1] Yes [2] No

30. Have you ever been hospitalized?

[1] Yes [2] No

31. If you were hospitalized, please indicate the reason here.

_____ ___

32. If you are female, have you ever had any menstrual irregularities?

[1] Yes [2] No

33. If you are female, are you currently pregnant?

[1] Yes [2] No [9] Don't know

34. Do you have a family history of any of the following medical problems?

	Yes	No	If yes, relation to you	
a. Liver Disease	[1]	[2]	_____	[___]
b. Neurological disease	[1]	[2]	_____	[___]
c. Kidney disease	[1]	[2]	_____	[___]

Appendix H
Physical Examination

ID Number ___ ___ ___

<u>Vital Signs</u>

35. Pulse Rate ___ ___ ___ Beats per Minute

36. Respiratory Rate ___ ___ ___ Breaths per Minute

37. Blood Pressure ___ ___ ___/___ ___ ___ mmHg

38. Weight ___ ___ ___ . ___ Pounds

39. Height ___ ___ ___ cm

40. Folic Acid Level ___ ___ . ___ NG/ML

	Normal	Abnormal	Not Checked	Comment	
Skin					
41. Color	[0]	[1]	[9]	________________	[___]
42. Texture	[0]	[1]	[9]	________________	[___]
43. Lesions	[0]	[1]	[9]	________________	[___]
44. Hair	[0]	[1]	[9]	________________	[___]
45. Nails	[0]	[1]	[9]	________________	[___]
Eyes					
46. Vision	[0]	[1]	[9]	________________	[___]
47. Conjuctiva	[0]	[1]	[9]	________________	[___]
48. Sclera	[0]	[1]	[9]	________________	
49. Extraocular Move	[0]	[1]	[9]	________________	[___]
50. Orthal. Exam	[0]	[1]	[9]	________________	[___]
51. Color Blind	[0]	[1]	[2]	________________	[___]

Ears

52. Auricules	[0]	[1]	[9]	_____	[___]
53. Canals	[0]	[1]	[9]	_____	[___]
54. Drums	[0]	[1]	[9]	_____	[___]
55. Auditor Acuity	[0]	[1]	[9]	_____	[___]

Nose

56. Mucosa	[0]	[1]	[9]	_____	[___]
57. Septum	[0]	[1]	[9]	_____	[___]
58. Sinus Tenderness	[0]	[1]	[9]	_____	[___]

Mouth

59. Lips	[0]	[1]	[9]	_____	[___]
60. Buccal Mucosa	[0]	[1]	[9]	_____	[___]
61. Gums	[0]	[1]	[9]	_____	[___]
62. Teeth	[0]	[1]	[9]	_____	[___]
63. Tongue	[0]	[1]	[9]	_____	[___]
64. Pharynx	[0]	[1]	[9]	_____	[___]

Neck

65. Thyroid	[0]	[1]	[9]	_____	[___]
66. Trachea	[0]	[1]	[9]	_____	[___]

Lymph

67. Cervical	[0]	[1]	[9]	_____	[___]
68. Axillary	[0]	[1]	[9]	_____	[___]
69. Epitrochlear	[0]	[1]	[9]	_____	[___]
70. Inguinal	[0]	[1]	[9]	_____	[___]

Thorax/Lung

71. Breathing Pat.	[0]	[1]	[9]	_____	[___]
72. Breathing Effort	[0]	[1]	[9]	_____	[___]
73. Breathing Sound	[0]	[1]	[9]	_____	[___]
74. Shape of Chest	[0]	[1]	[9]	_____	[___]
75. Fremitus	[0]	[1]	[9]	_____	[___]
76. Percussion Note	[0]	[1]	[9]	_____	[___]
77. Breath Sound	[0]	[1]	[9]	_____	[___]
78. Adventit. Sound	[0]	[1]	[9]	_____	[___]

Cardiovascular

79. Carotid Pulse	[0]	[1]	[9]	_____	[___]
80. JVP	[0]	[1]	[9]	_____	[___]
81. Vein Quality	[0]	[1]	[9]	_____	[___]
82. Apical Impulse	[0]	[1]	[9]	_____	[___]
83. Heart Sounds	[0]	[1]	[9]	_____	[___]
84. Heart Murmers	[0]	[1]	[9]	_____	[___]

Abdomen

85. Shape	[0]	[1]	[9]	_____	[___]
86. Scars	[0]	[1]	[9]	_____	[___]
87. Percussion Note	[0]	[1]	[9]	_____	[___]
88. Tenderness	[0]	[1]	[9]	_____	[___]
89. Palpable Struc.	[0]	[1]	[9]	_____	[___]

Peripheral Vascular

90. Skin Color	[0]	[1]	[9]	_____	[___]
91. Periph. Pulses	[0]	[1]	[9]	_____	[___]
92. Edema	[0]	[1]	[9]	_____	[___]
93. Varicose Veins	[0]	[1]	[9]	_____	[___]

Neurologic

94. Cranial Nerves	[0]	[1]	[9]	_____	[___]
95. Gait	[0]	[1]	[9]	_____	[___]
96. Tandem Walking	[0]	[1]	[9]	_____	[___]
97. Toe Heel Walking	[0]	[1]	[9]	_____	[___]
98. Hops	[0]	[1]	[9]	_____	[___]
99. Knee Bends	[0]	[1]	[9]	_____	[___]
100. Muscle Bulk	[0]	[1]	[9]	_____	[___]
101. Strength	[0]	[1]	[9]	_____	[___]
102. Coordination	[0]	[1]	[9]	_____	[___]
103. Involuntary Move.	[0]	[1]	[9]	_____	[___]
104. Pain	[0]	[1]	[9]	_____	[___]
105. Light Touch	[0]	[1]	[9]	_____	[___]
106. Vibration	[0]	[1]	[9]	_____	[___]
107. Position	[0]	[1]	[9]	_____	[___]
108. Descriminative S.	[0]	[1]	[9]	_____	[___]
109. Reflexes	[0]	[1]	[9]	_____	[___]

Mental Status

110.	Appearance Beh.	[0]	[1]	[9]	[___]
111.	Speech/Lang.	[0]	[1]	[9]	[___]
112.	Mood	[0]	[1]	[9]	[___]
113.	Thought/Percep.	[0]	[1]	[9]	[___]
114.	Memory/Attent.	[0]	[1]	[9]	[___]
115.	Higher Cognitive	[0]	[1]	[9]	[___]
116.	Sleep Disturb.	[0]	[1]	[9]	[___]
117.	Comments				[___]

Appendix I

Instructions for the Researchers to perform NBT

Two and Seven Test

"This test is called the 2 & 7 Test and this is the practice section. What you do here is find all the 2's and all the 7's and cross them out like this."

(Demonstrate: subject should put a short slash through the target numbers. Circling numbers or putting "X's" through them is not allowed).

"Go from the left to the right in order and then drop to the next line and keep doing the same thing. Now you probably will not finish this whole thing; that's okay. After you have been working for a little while, I am going to say "next". When I say "next", I want you to stop wherever you are and drop under the solid line to the next part starting again at the left marking out only 2's and 7's."

Administer the practice observing the subject's performance. The examiner should allow 15 seconds to elapse then say "next".

"Good. Now here is the real test. You are going to start up here at the top and find all the 2's and 7's. Every time I say "next", you drop down to the next section and do the same thing. I want you to go as fast as you can without missing any of the 2's or 7's."

Again , each section is 15 seconds long. It is important that the subject works from left to right and does not skip around between lines. Should the subject start marking on an incorrect segment, the examiner redirects him or her to the appropriate slot without restarting the fifteen second interval.

Symbol Digit

"Now here is a digit, find its number and write the number below it. Try the next symbol. And here is another one, complete its number."

If the subject has completed the first three squares correctly, say: "Starting from here

you will continue this task until I tell you stop. Do not skip any symbol. Work quickly."

Say: "Start now."

Start the stopwatch. At the end of 90 seconds, say: "Stop."

Stroop

This is a test of how fast you can read the words on this page. After I say begin, you are to read the words on this page. After I say begin, you are to read down the columns starting with the first one (point to the leftmost column) until you complete it (run hand down to the left most column) and then continue without stopping down the remaining columns in order (run your hand down the second column, then the third, fourth and fifth columns). If you finish all the columns before I say "Stop", the return to the first column and begin again (point to the first column). Remember do not stop reading until I say "Stop" and read out loud as quickly as you can. If you make a mistake I will say "no" to you. Correct your error and continue without stopping. Are any questions? Instructions may be repeated or paraphrased as often as necessary so theat the subject understand what is to be done. Then continue. "Ready? Then begin." As the subject starts , begin a stopwatch. After 45 seconds, say: "Stop." Circle the item the subject is on. If the subject finished the entire page and began again, put a one by the circle. Turn the page.

The instructions for the second page are identical, except the first sentence reads: "This is a test of how fast you can name the colors on this page. You will complete this page as you did the previous page, starting with the first column. Remember to name the colors out loud as quickly as you can."

If the subject has had any trouble following the instructions, they should be repeated in their entirety. As with the first page, the subject should be allowed 45 seconds.

At the beginning of the third page, The following instructions should be used:

"This page is like the page you just finished. I want you to name the color of the ink

the words are printed in, ignoring the word that is printed in each item. For example, (point to the first item of the first column), this is the first item: what would you say? If the subject is correct go on with the instructions. If incorrect, say: No, that is the word that is spelled there. I want you to say the color of the ink the word is printed in. Now (pointing to the same item) what would you say to this item? That's correct (point the second item), what would the response be to this item? If correct, proceed; if incorrect, repeat above as many times as necessary until the subject understands or it becomes clear that it is impossible to go on. "Good you will do this page just as the others, starting with the first columns as you can. Remember if you make a mistake, just correct it and go on. Are there any questions?" (as with the other two pages, the instruction can be repeated or paraphrased as often as necessary). "Then begin" (Time for 45 seconds, then say: "Stop". Circle the item the subject is on.

Appendix J

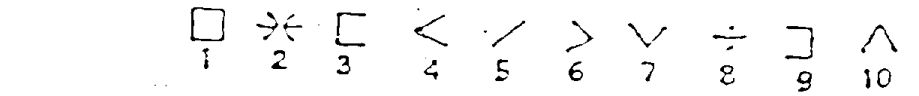
ID #_____

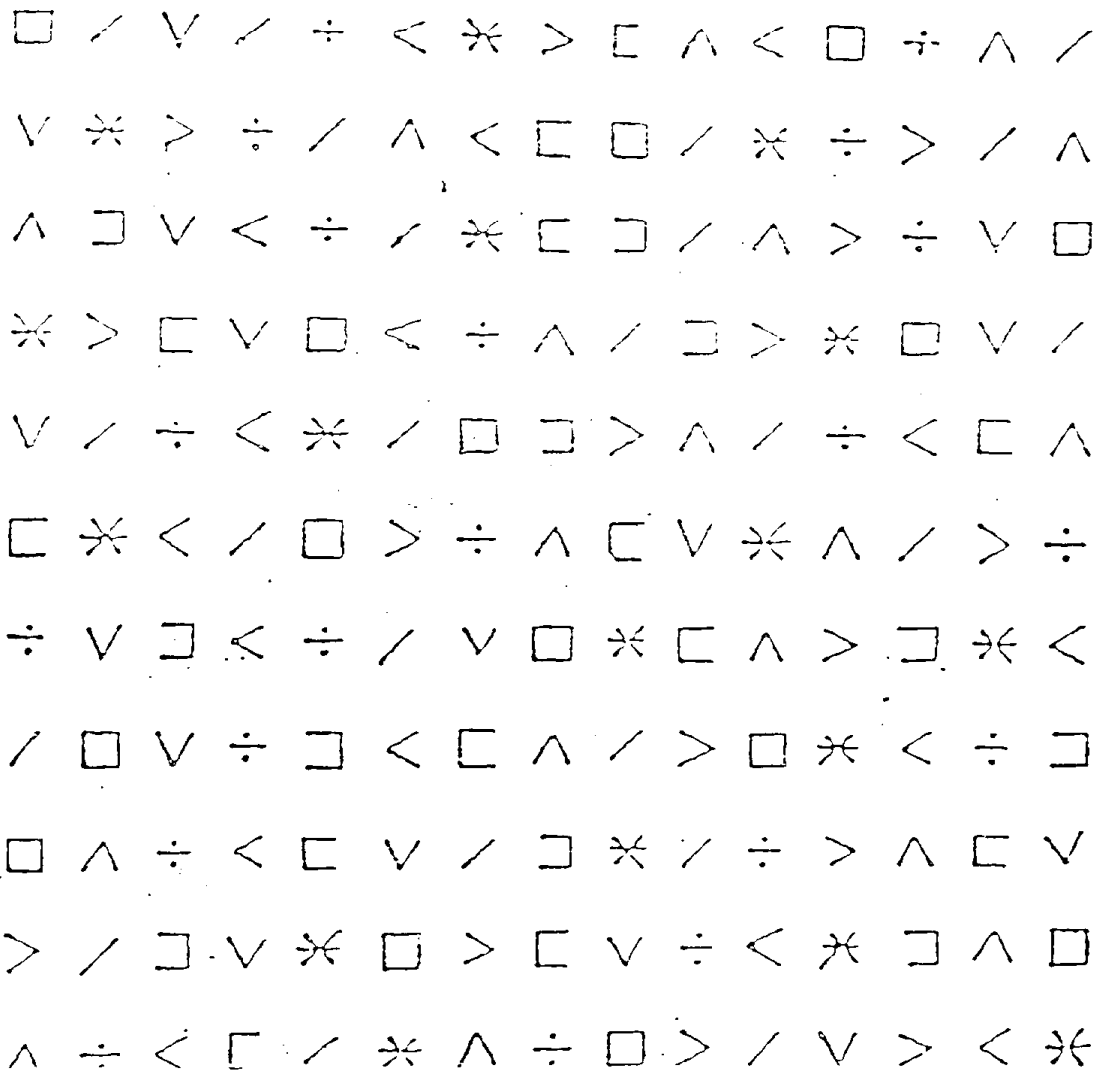
2 & 7

Appendix K

Symbol Digit

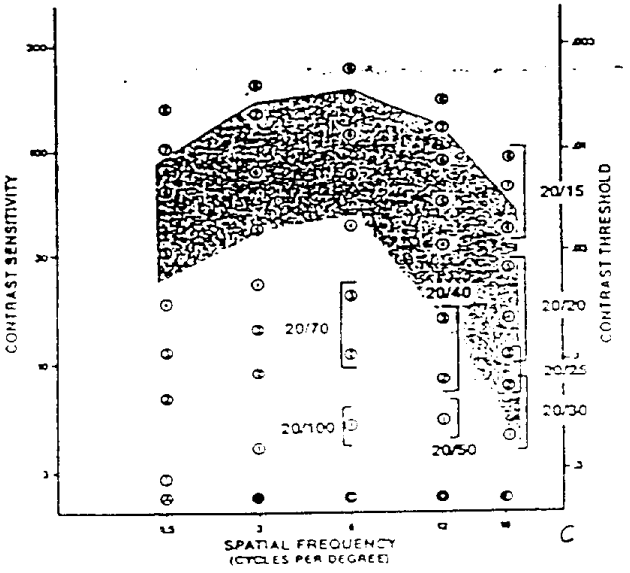
Name _____ ID No. _____ Date _____





Appendix L

Vistech



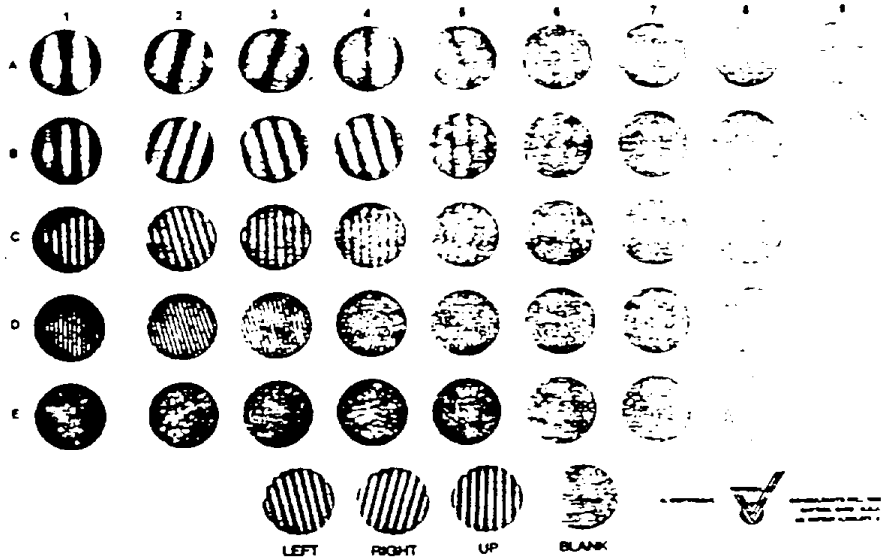
OBSERVER NAME _____ DATE _____

VCTS® SYSTEM USED _____ TESTING DISTANCE _____

CONTRAST SENSITIVITY EQUIVALENT ACUITY RIGHT _____ LEFT _____

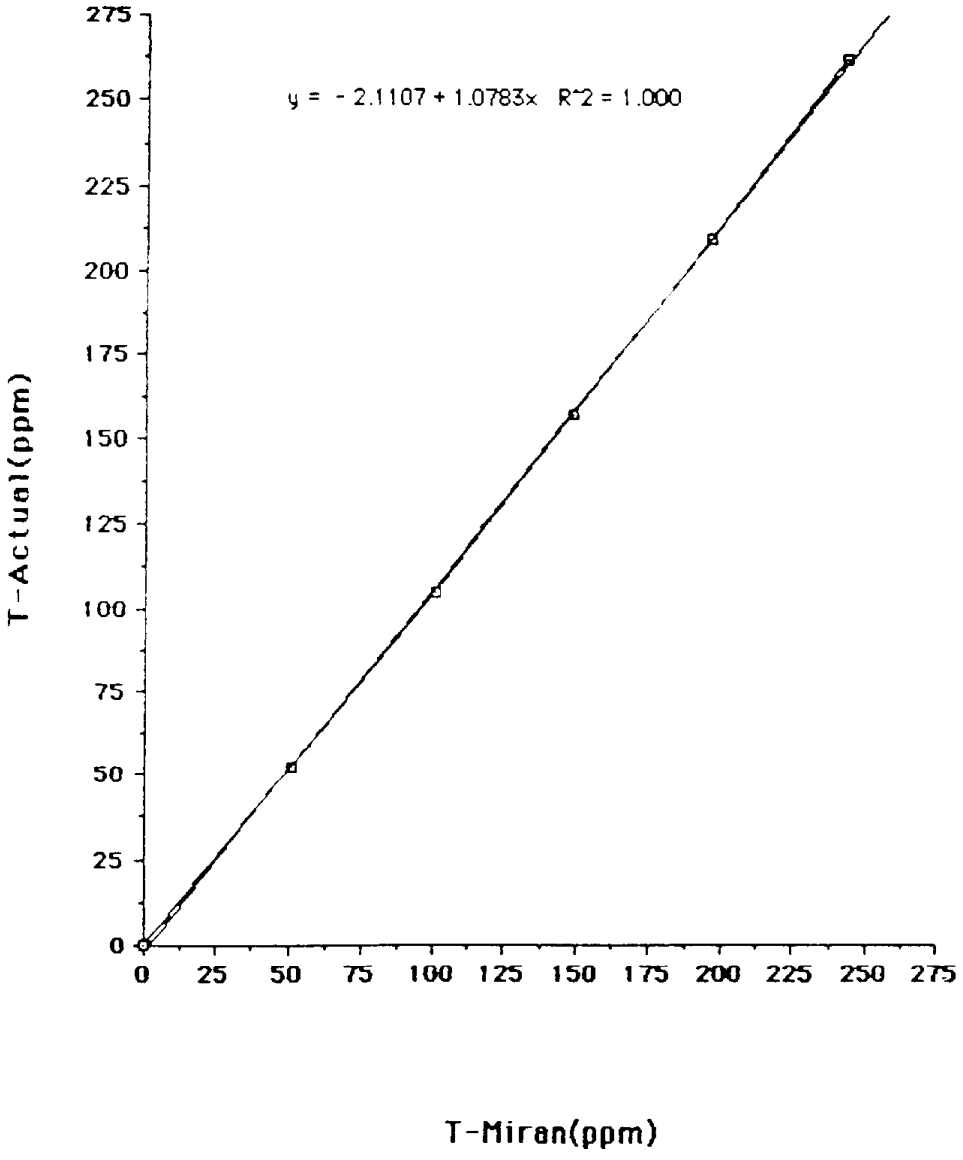
COMMENTS _____

VISION CONTRAST TEST SYSTEM



Appendix M

Tedlar Bag Calibration - Miran/MEOH



Appendix N

Development and Validation of Head Space-Gas Chromatographic Method for Methanol

BACKGROUND

Methanol is quantified by analyzing the headspace content of heated blood samples using GC/FID. Described is a method for the determination of low level blood-methanol by GC/FID. 100 µl of serum and 100 µl of acetonitrile solution (internal standard) are introduced in a headspace vial containing 1 gm of NaCl. The sample is heated for a period of time during which the volatile components of the sample diffuse into the vial's atmosphere (known as headspace). 1 ml of headspace is transferred via heated transfer line to the GC. Methanol values are determined by a calibration method using aqueous methanol standards.

INSTRUMENTATION

1. Headspace Autosampler (Tekmar 7000)
2. Carrousel with 50 sample capacity (Tekmar 7050)
3. Gas Chromatograph/FID (Hewlett Packard)
4. PC (A/D board installed) (CTX)
5. Chromatographic Software (EZ Chrom Software)
6. Printer (Citizen 200GX)
7. Hydrogen (purity 99.99%)/Air (21% oxygen and 79% nitrogen) are used for FID, and Nitrogen (purity 99.999%) is used as carrier gas and pressurization gas.

MATERIALS

1. Variable volume pipets and disposable tips. Sizes 5 ml and 200 ul.
2. 22 ml headspace vial.
3. 20 ml scintillation vial(glass).
4. 20 mm rubber stopper.
5. 20 mm metal seal
6. Cap crimper (20mm neck)

REAGENTS

1. Methanol - AR grade (Fisher).
2. Acetonitrile - AR grade (Fisher).
3. Sodium Chloride - Ar grade (Aldrich)
4. Water - deionized to approximately 18 M /cm quality (Milipore Corporation)

STANDARDS, CALIBRATORS, AND QUALITY CONTROLS

1. Methanol optima grade (density = 0.7914 g/ml at 20°C), 99.999% purity (Fisher product) is used for preparation of calibrators and in-house QCs.

2. Acetonitrile optima grade (density = 0.7857 g/ml at 20°C), 99.999% purity (Fisher product) is used as internal standard.

3. Calibrators:
Every two weeks calibrator stock solution I with methanol concentration of 3.94 g/L is prepared by spiking 100 µl of pure methanol to 20 ml of water. Then prepare stock solution II with methanol concentration of 152 mg/L by spiking 200 ul of stock into 5 ml of water.
Working calibrators with methanol concentration of 2.98, 5.84, and 11.3 mg/L are prepared by spiking 100 µl, 200 µl, and 400 µl of stock solution II in 5 ml water. Water is used as the blank. Stock solution of 3.96 g/L is stored in -4°C refrigerator.

4. Internal Standard
Internal standard is prepared fresh daily due to the instability of acetonitrile. First prepare a stock solution with acetonitrile concentration of 3.91 g/L by spiking 100 µl of pure reagent in 20 ml of water. Then spike 50 µl of stock into 20 ml of water. The final concentration of acetonitrile yield 9.7 mg/L.

5. Methanol in-house spiked QC:
QC's with methanol 3.94 mg/L are prepared by diluting 200 µl of stock solution I to a final volume of 200 ml. Mix the solution well and transfer in portions to 20 ml glass scintillation vial. Place vials in a ziplock plastic bag and store them in -4°C refrigerator. Calculate the actual concentration and assay to determine the measured concentration. The stability has been established to be three months.

PROCEDURE

1. Serum samples to be assayed are in 7 ml polyethylene screw cap vials, labeled, and stored in -80°C freezer. QC's are stored in glass scintillation vials at - 4°C. Frozen samples are brought to room temperature to be thawed. Do not heat the samples and keep the vials capped tightly. To accomplish thorough mixing, each sample will be vortexed before sampling.

2. Place 22 ml headspace vials in the vial holder. Into each vial place 1 gm Sodium Chloride salt with the measuring spoon. 100 µl of sample and 100 µl of Internal standard are introduced to the wall of the vial. The vial is then capped and sealed.

3. Instrument Set-up

The GC and Autosampler is always on. There is 20 ml/min Nitrogen flow going through transfer line to the GC. Hydrogen flow rate is set at 30 ml/min and Air flow rate at 425 ml/min.

Set up the instrumental parameters in the following order:

a.-Autosampler

At stand by mode:

1. Press F1 for Method
2. Activate Method 1
3. Press F3 : Edit Parameter
4. Platen Temperature: 95°C
5. Platen Eq. : 0.10
6. Sample equilibration time : 5.0
7. Vial size : 22 ml
8. Page down
9. Mixer : ON
10. Mix : 0.50
11. Mix Power : 1
12. Stabilize : 0.20
13. Page down
14. Press. : 0.50
15. Press. Eq. : 0.20
16. Loop : 0.25
17. Loop Eq. : 0.20
18. Inject : 1.00
19. GC Cycle Time : 8.00
20. Page down
21. Exit
22. Press A/S
23. Determine the slot number for the sample #1 to #X
24. Exit
25. Press Auto.

Wait the ready sign

Note: For further manipulation refer to Tekmar Instruction Manual section 9.

b.- Gas Chromatograph

1. Oven Temp. : 130°C
2. Initial Value: 130°C
3. Rate: 0 DEG/MIN
4. final Value: 130°C
5. Final Time: 0.00 MIN
6. Injection A : 200°C
7. Detector A : 200°C
8. Equil. Time: 3 MIN
9. Detec. : ON
10. Press FID ignitor button; it should pop and the signal A should read between 20-30.

c.- EZ Chrom Software

1. From C-drive go to window and click on CHROMATOGRAPHY.
2. Click on EZ CHROM box
3. Log in
   User: Hosna
   password: methanol

User and password should be typed exactly as it appears above.

4. Click on HP5890 to open Data Acquisition mode.
5. Under FILE click on OPEN METHOD.
6. Click on meoh.met.
7. Click on OK.
8. Under FILE open NEW BATCH.
9. A dialogue box appears.
10. Type SAMPLE ID.
11. Click on AUTO INCREMENT for numbering the sequence automatically.
12. Click on METHOD NAME and chose the corresponding method.
13. Type the subject ID with the date for the FILENAME
    example:
    subject 400, date: november 12, 1993

    Filename : 400111293

14. Click on AUTO INCREMENT.
15. Type 1 for SAMPLE AMOUNT.
16. Type 1 for INTERNAL STANDARD AMOUNT. (if none used, type 0)
17. Type 1 for MULTIPLIER.
18. Type applicable numbers for NUMBER OF RUNS.
19. Click on OK.

A batch spreadsheet containing the entered information appears.

20. Type the level of calibrators and their corresponding ID, along with the sample ID's.accordingly.
21. Under FILE go to SAVE BATCH as subjectID/mon/day/year.seq
22. Under METHOD go to peak table.
23. Update the concentration of new calibrators at their level
24. Under METHOD go to the ACQUISITION SETUP
    Check the followings:
    Channel : A, Sampling frequency : 5.00
    Run Time: 8 min Delay: 0.00 min
    Channel Statues: on Trigger: contact closure
25. Save METHOD as meoh.met.
26. Under ANALYSIS go to ANALYSIS OPTION and chose PRINT.
29. Click on BATCH RUN to go to BATCH ACQUISITION
30. Click on CATALOG and select the corresponding BATCH.SEQ.
31. Confirm overwriting Existing files.
32. Type corresponding numbers for START RUN #, AND END RUN #
33. START the analysis.

34. Turn on the PRINTER
35. The computer is ready for data collection and is waiting for trigger

4. Procedure for analysis:

a.- The calibrators, QC's and samples are prepared in duplicates. Calibrators are placed in slots numbered 1 to 10. QC's and samples are placed in slots numbered from 11 to 50.

b.- If the number of samples exceed 40, after the sample in the 50th position is analyzed remove the samples from the slot 1 to 50. Place new prepared sample and instruct the Autosampler to start analysis from slot # 1 to #X.

c.- Sample Analysis

1. Place Calibrators, QCs and the samples in the carrousel and program the autosampler to analyze only from #1 to #10.
2. Press START on the Autosampler. Press YES if GC applicable times are correct.
3. Analyze the calibrators and QC's first. View the calibration curve. If R>0.98, and the slope is between 20-35, determine the value of the QC's. If QC's values fall within the given range, proceed with the batch analysis. Otherwise recalibrate.
4. After 12 sample (6 pairs) 1 QC and 1 calibrator (mid) will be analyzed. If the QC values are out of the range and calibrator is reproduced, allow computation. If both the calibrator and QC are out of the required range and the batch run is completed, recalibrate on new calibrator response for previous sample. 12

DATA MANIPULATION

After the batch run ends, data manipulation is done through Batch reprocessing in following:

1. Under METHOD go to the PEAK TABLE and type 2 for INTERNAL STANDARD ID.
2. Select EXPORT as ANALYSIS OPTION.
3. Open Excel file
4. Under BATCH go to BATCH REPROCESS, reprocess from #8 to #x.

Calculated Methanol concentration based on the calibration curve for the samples will be transported to Excel as MEOH.AIS file.

QUALITY ASSURANCE:

During an analysis, the QC values reported must be within 2 standard deviation of the previously determined mean for that QC. Any value greater than 2 standard deviation will require recalibration and re-analyzing the previous samples. Repeated calibrators should be within 10%.

SHUT DOWN:

Turn off in sequence
1. Printer
2. Computer
   a: Log off
   b: End Window Session
3. Detector
4. Turn Off Hydrogen/Air gas.

TROUBLE SHOOTING

Several possible causes exist for overall poor sensitivity and reproducibility of the chromatogram. A number of steps can be taken to prevent such instances.

1. For poor peak shape or tailing
   a)-adjust line and valve temperature.
   b)-increase loop equilibration time.
   c)-verify the column flow rate in the optimum range.
   d)-verify GC oven condition.

2. For poor sensitivity or obtaining no peak
   a)-perform leak check diagnostics (Tekmar manual, section 4).
   b)-verify the column flow rate.
   c)-verify that the needle is not bent or broken.
   d)-verify the function of A/D board by checking the preview.
   e)-verify the attenuation.

3. For carryover
   a)-check the standby flow rate at the vial needle.
   b)-potential liquid contamination on septum or needle-- use lower mix time, clean needle and lines by running blanks.

4. For retention variation
   a)-verify the oven temperature.
   b)-check the column flow rate, verify transfer line back pressure.

5. For baseline drift

a)-check the back pressure for the carrier gas. Potential valve operation-- possible valve block-- remove the valve and wash it.
b)-possible leak in the transfer line.

For more detailed information refer to the trouble shooting sections of Tekmar 7000 Instruction Manual and Hewlett Packard reference manual. If more technical information needed, contact

1. Tekmar Services Department at (800) 874-2004
2. Hewlett Packard at (800) 752-0900

REFERENCES:

Effect of Methanol Vapor on Human Neurobehavioral Measures

Mary r. Cook, Fred J. Bergman, Harvey D. Cohen, Mary M. Gerkovich, Charles Graham, Roger K. Harris, and Linda G. Siemann.

DOCUMENTATION SUMMARY

Headspace Measurement of Blood Methanol by Gas Chromatography with a Headspace Autosampler

The automated headspace measurement of methanol in blood by GC eliminates problems associated with liquid injection: such as, use of pre-column, which has to be changed often, accumulation of blood protein in the injector, injection of large volume of samples to increase sensitivity. In addition, the use of an autosampler leads in obtaining more precise and reproducible results while it offers convenience in sample handling.

The 22 ml vials are standard size vials and they were adequate for our analysis. Acetonitrile was chosen as internal standard due to its similar physical and chemical characteristics with methanol.Injection size of the headspace content was 1 ml that maximizes the sensitivity at the levels of interest and at the same time minimizes overloading the column. Sample sizes of 100 μl and 200 μl were compared. (Table 1.) 100 μl sample size determined to be adequate for blood methanol.

To evaluate the method, prepared calibrators in water and Bovine serum with methanol concentration of 0, 0.45, 3.34, 6.52, 12.61 mg/L. Single injection of duplicates were made at each concentration for both, serum and aqueous standards. The slope value for both, serum and aqueous standard curves (Table 2.) were the same; however, the blank values for serum standards were much higher than for aqueous ones. Serum blanks varied in their methanol area counts and air, water and internal standard blanks were constant (Table 3.)

Maximum sensitivity for 200 μl of analyte and 100 μl of internal standard was achieved at bath temperature of 95°C (Table 4.), addition of 1 gm of NaCl (Table 5.) and sample equilibration time of 5 min.(Table 5.) Intra-assay precision for serum methanol (conc.=3.06 mg/L, and n=10) has a SD of 0.287, and a CV of 9.4%; for aqueous methanol (conc.= 3.34 and n= 18), SD is 0.149 and CV of 3.9%. (Table 7.) Paired precision was determined and calculated in serum from subjects run in duplicates. (Table 9.)

To determine accuracy, 16 specimen were analyzed and compared to their results determined 2 years ago. (Table 9.) There were only 3 out of 16 that were not in agreement with the previous results. The mean bias and the paired t-test were determined.

1. SAMPLE SIZE

Table 1. 200 µl and 100 µl of 2 mg/L MeOH-serum and their corresponding real count.

SAMPLE SIZE	N	MEOH AREA COUNT	SD	%CV
100 µl	4	23,069	1,468	6.4
200 µl	4	29,852	882	3.0

Batch 070193.seq, date: 07,01, 1993

2. STANDARD CURVE AND BLANK VALUES

Evaluated the External and Internal Standard Curve for serum-methanol standards and aqueous-methanol standard. Table 1 represents the slope, and the intercept values.

Table 2. Internal Standard Curve

MEDIUM	DATE	#'S OF STD	SLOPE	INTERCEPT
SERUM	07/08/93	4	27.89	3.004
SERUM	07/12/93	5	24.83	1.1650
SERUM	08/16/93	5	24.64	0.4722
MEAN SD %CV			25.8 1.824 7%	1.5471 1.308 85%
WATER	08/16/93	5	25.33	0.5516
WATER	09/14/93	4	23.15	0.2473
WATER	09/13/93	4	23.04	0.4177
MEAN SD %CV			23.84 1.292 5%	0.4055 0.1525 4%

Serum internal curve and water internal curve run on August 16, 93 demonstrate that the slope values for serum and water on the same day are not different. Aqueous blank area ratio is 0.025 and serum blank area ratio is 0.0347.

Table 3. Evaluation of Blank
(batch 080293.seq; Aug. 8,93)

SAMPLE ID	N	MEAN VALUE	SD	%CV
AIR	5	2,611	268	12
WATER	5	2,911	341	14
ACN	5	2,645	325	15
SERUM	4	7,284	212	3.0
SERUM(1)	2	6,183	33.2	0.54
SERUM(2)	2	1,812	16.3	0.90
SERUM(3)	2	3,031	26.9	0.88

Serum 1, 2, and 3 are aliquotes of the same pool.
(1). Serum sample removed from the -80°C freezer, kept in the -4°C refrigerator and analyzed after a week. (July 12, 93)
(2)&(3). Serum sample removed from the freezer thawed and analyzed.
(2). August 18, 93 and (3). August 12, 93.

2. VARIABLE AFFECTING RESPONSE:

Headspace response is related to the concentration of the analyte. Maximum sensitivity was achieved by:

a- Increasing bath temperature; raising bath temperature enhances the sensitivity by saturating vapor pressure of the pure trace component. Table 4. shows that by increasing bath temperature the peak area counts of Methanol increase and they are directly proportional to each other.

Table 4.

BATH TEMPERATURE	MEOH PEAK AREA	N	SD	%CV
75°C	17,932	4	672.65	3.8
85°C	23,382	4	493.31	2.1
95°C	25,717	4	746.44	2.9

200 µl of 2 mg/L serum-methanol + 100 µl 6.46 mg/L Acetonitrile + 1 gm NaCl.(Batch ID 063093.seq, date: June 30, 93)

b- Addition of Salt; ions of dissolved salt in the aqueous medium and increase the concentration of the volatile trace components in the headspace. The data in Table 5. clearly shows that the addition of salt increase the sensitivity.

Table 5.

gm of SALT ADDED	MEOH PEAK AREA	N	SD	%CV
1 gm	25,717	4	746.44	2.9
0	19,846	4	689.14	3.5

200 µl of 2 mg/L serum-methanol + 100 µl 6.46 mg/L ACN.
(Batch ID 063093.seq, date: June 30, 93)

c- Sample Equilibration Time; the concentration of analytes increase in the headspace with time. After sufficient time has passed, the concentration of analytes reaches equilibration. Degradation can occur after longer heating time.
Table 6. demonstrate the effect of sample equilibration time on serum-methanol sample. Methanol peak area for samples analyzed at 5 min compared with the analysis of original sample at 2 min and 10 min indicates that the maximum sensitivity has been achieved. Further, SD and %CV values show that 5 min sample equilibration results in better precision.

Table 6.

SAMPLE EQ. TIME	MEOH PEAK AREA	N	SD	%CV
2 min	27,480	4	1,977	7.2
5 min	29,853	4	882	3.0
10 min	28,153	4	1,667	5.9

200 µl 2 mg/L serum-methanol + 100 µl 6.46 mg/L ACN + 1 gm NaCL
(Batch ID 070193.seq, date: July 1, 93)

3. PRECISION

a)-Intra-assay and interassay precision

Table 7.

INTRA-ASSAY PRECISION	CONC. (actual)	MEAN	N	SD	%CV
1. serum-MeOH (1)	3.02mg/L	3.06 mg/L	10	0.287	9.4
2. water-MeOH (2)	3.34mg/L	3.87 mg/L	18	0.149	3.9
INTER ASSAY PRECISION					
1. serum-MeOH (3)	3.02mg/L	3.57 mg/L	5	0.911	25.5
2. water-MeOH (4)	3.34mg/L	3.55 mg/L	6	0.220	6.2

1.QC-seq.;Aug.13,93. 10 analysis of single preparation.
2.calib-seq.; Aug.18,93.18 analysis of single preparation.
3. 5 analysis, 5 single prep.(duplicate sampling, stored at -80°C)
4. 2 analysis, 2 single prep.(duplicate sampling, stored at -4°C)

b)- different sample preparation:
Examined the precision between two different sampling procedure. Preparation 1.: in a set of vials added salt, then in each spiked the sample, followed with the addition internal standard, and finally capped and sealed. Preparation 2.: in a single vial added salt, spiked sample and internal standard, capped, sealed and proceeded with the next vial.(Table 8.)

Table 8.: sd and %cv for n=10 for prep. 1 and prep. 2

PROCEDURE	N	AREA COUNT MEAN	SD	%CV
PREPARATION 1	10	19,193	518	2.7
PREPARATION 2	10	18,766	728	3.9

(batch 080293.seq, Aug.2,93)

c)- Paired Precision : For a series of subject specimen run in duplicates calculated the paired precision. Paired Precision for subjects (1) is 0.051 mg/L and for the subjects(2) is 0.129 mg/L. Table 9. shows only the mean values for subject.

d)- Accuracy: To determine accuracy, 16 subject specimen were compared to their values determined 2 years ago. The mean bias for the subject specimen is calculated to -0.50 mg/L, and the paired t-test is as following

determination	n	mean	sd	se-mean	t
current:	16	3.966	1.730	0.433	9.17
previous:	16	4.471	1.645	0.411	10.87

Table 9.

SAMPLE ID	ISTD-MG/L MEOH	PREVIOUS DATA	DIFFERENCES
403-1-11:15(1)	3.26	2.81	+0.45
403-1-12:15(1)	4.25	3.96	+0.29
403-1-04:45(1)	2.67	3.55	-0.88
403-1-05:45(1)	1.89	1.89	0.00
403-1-12:00(2)	4.33	2.81*	+1.52
402-1-11:45(2)	5.85	5.89	-0.04
402-1-12:15(2)	6.12	5.57	+0.55
402-1-10:15(2)	5.10	5.40	-0.30
402-1-09:00(2)	2.41	2.29	+0.12
403-1-10:45(2)	4.05	5.04	-0.87
402-1-08:30(2)	0.53	3.45*	-2.92
402-1-11:15(2)	6.41	7.62	-1.21
403-1-13:00(2)	4.85	6.59	-1.74
402-1-09:45(2)	4.25	4.15	+0.10
301-1-13:00(2)	5.69	6.08	-0.39
404-1-09:45(2)	1.80	4.44*	-2.64

1. Calib.seq, August 18,93.; paired precision for ISTD= 0.051 mg/L.
2. 082093.seq, August 20,93; paired precision for ISTD= 0.129 mg/L.
* High values.

Improved Enzymic Assay for Serum Formate with Colorimetric Endpoint

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Abstract

The fluorometric assay for formate in serum was modified by pretreating samples with acetonitrile (1:1) precipitation; substituting *p*-iodonitrotetrazolium violet (INT) for resazurin; and by combining the cofactor (NAD), coupled enzyme (diaphorase), and secondary substrate (INT) into one reagent. Formate is oxidized by formate dehydrogenase producing NADH which reduces INT via diaphorase to a visible red-colored endpoint that can be measured on a spectrophotometer at 500 nm. Previous problems with fluorometric endpoint methods are eliminated when using this modified procedure: calibration is linear rather than nonlinear, blanking is rarely needed due to the acetonitrile sample preparation; dynamic range is expanded up to 10-fold; a simple spectrometer rather than a fluorometer is used; and the number of steps is reduced. The method is demonstrated to be linear, specific, sensitive, precise, and accurate.

Introduction

During methanol poisoning, criteria used in the decision to initiate hemodialysis are varied and have included combinations of the following: history of ingestion, amount ingested (>30 mL), ocular symptoms or signs, acidosis (decreased bicarbonate, pH, or widened anion gap), elevated osmolar gap, and methanol concentrations greater than 500 mg/L or 1000 mg/L (1-5). Formate is the mediator of the late toxic effects, accounting for more than 50% of the drop in bicarbonate*** and direct retinal injury (6-8). Therefore, the combination of a history of methanol ingestion, methanol measurement or a widened osmolar gap, and the presence of a metabolic acidosis have been used as the determinants for initiating hemodialysis (4,5,8,9). More recently, concentrations of the toxic metabolite (formate) in serum have been used as a more direct measure of toxicity (7,10-12).

This paper presents a convenient method for serum formate based on modifications of the formate dehydrogenase (FDH) (EC1.2.1.2.)-diaphorase procedure presented by Makar (13,14). In this procedure, a visible chromagen is substituted for a fluorescent endpoint, several steps are eliminated, the number of separate reagents are reduced, and storage and stability of reagents are described.

Experimental

Reagents

Lyophilized nicotinamide adenine dinucleotide-diaphorase (NAD-diaphorase), *p*-iodonitrotetrazolium violet (INT), formate dehydrogenase, and sodium formate were all obtained from Sigma Chemical Co. Acetonitrile, Na_2HPO_4, and KH_2PO_4 were of analytic grade and reverse osmosis deionized water was used. Phosphate buffer (100 mmol/L, pH 6.0) was prepared by mixing 100 mmol/L KH_2PO_4 and 100 mmol/L Na_2HPO_4 (5:1). Buffered NAD-diaphorase was prepared by adding 150 mL of buffer to one bottle of lyophilized NAD-diaphorase (Sigma, 940-3). Buffered NAD-diaphorase-INT was prepared by adding 140 mg INT (Sigma, 940 SI P) to 140 mL of buffered NAD-diaphorase, and FDH (Sigma, F-8753) was received lyophilized. Five lots were used having specific activities of 0.4 to 1.0 units/mg solid (0.4 to 1.8 units/mg protein). Portions (5 to 15 mg) were weighed into 1.5-mL glass vials and stored at −70° C (Sigma indicates storage at less than 0° C is sufficient.) One vial was used for each batch analysis. Each was reconstituted with about 100 to 200 μL/mg buffered NAD-diaphorase (5° C) and kept in an ice bath during analysis. Serum and aqueous standards (10 mL each) were supplemented with stock aqueous sodium formate solutions containing 10.0 or 1.0 g/L to produce concentrations ranging from 0 to 400 mg/L with less than 1% volumetric alteration of the matrix. Working reagent and final reaction (cuvette) concentrations are shown in Table I.

Procedure

Serum samples and standards (100 μL) were vortexed (5 sec) with 100 μL acetonitrile in 1.5-mL polypropylene centrifuge tubes and centrifuged (14,000 g) for 2 min. Supernatants (100 μL) were added to 5-mL disposable plastic tubes containing 3.0 mL buffered NAD-diaphorase-INT. After mixing, 60 μL of FDH (5 units/mL at 5° C) were added. These were mixed and left at

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***To compare formate concentrations to meq/L of bicarbonate divide mg/L by 45.02 to give mmol/L.

room temperature for 10 min. Each preparation was subsequently aspirated into the sipper-cuvette of a Stasar III (Gilford) or similar single beam spectrophotometer. The instrument was zeroed on water and absorbances were read at 500 nm. Absorbances vs. concentrations of the standards were plotted. A direct procedure was also used for this study, wherein 20 μL of serum were added to the NAD-diaphorase-INT.

Reaction completeness and end product stability were assesed by measuring the change in absorbance from 1 to 55 min after the addition of FDH. Absorbance readings were taken at 30-sec intervals with the spectrophotometer connected to a CP 5000 Processor (Syva Co.) for both direct and pretreated sample procedures at several concentrations. Calibration response was determined using supplemented aqueous and serum matrices. The absorbance vs. concentration was assessed by least squares linear regression. Calibration response (absorbance mg/L, slope) was compared over a period of several months for both direct and acetonitrile pretreatment procedures. To assess recovery, additional formate was added to sera from patients overdosed on methanol to increase the concentration by 13 and 66 mg/L using the acetonitrile pretreatment procedure.

Sera containing formate (140 mg/L) were reassayed after supplementation with toxicologically significant concentrations of ethanol, methanol, or acetone (up to 5000 mg/L), with ethylene glycol (up to 100 mg/L), formaldehyde or acetaldehyde (up to 1000 mg/L), lactate (up to 900 mg/L, 10 mmol/L), and or acetonitrile (up to 600 μL reaction mix tube). Clear, lipemic, icteric, and hemolyzed samples from non-poisoned patients were also assayed. For these specimens, only 40 μL of supernatant from acetonitrile pretreated samples (containing 20 μL of serum) and 20 μL of serum added directly were used so that equal sample sizes would be compared.

Sera supplemented with 5, 10, 15, 25, and 140 mg/L were assayed in the acetonitrile pretreatment protocol for within-run precision. Frozen (−70°C) supplemented sera were assayed (25 and 140 mg/L) on 12 occasions over a three-month period for between-run precision. To estimate the accuracy of the new procedure, seven samples from actual methanol overdoses representing a range of 0 to 257 mg/L were assayed and sent for commercial analysis by headspace gas chromatography (GC) following the formation of methyl formate derivatives.

Buffered NAD-diaphorase and buffered NAD-diaphorase-INT were stored at 2° to 8°C. After six weeks, slopes and intercepts were compared by performing standard curves for stored and freshly prepared reagents. Buffered NAD-diaphorase was also reassessed after an additional 13 weeks of storage at 2° to 8°C.

Table I. Reagent and Cuvette Concentrations

Reagent	Commercial Form	Working Reagent Concentration	Cuvette Concentration
NAD*	Lyophilized	1.51 mmol/L** (1 g/L)	1.43 mmol/L
Diaphorase*	Lyophilized	800 U/L**	759 U/L
INT	Analytic grade powder	2.10 mmol/L** (1 g/L)	1.99 mmol/L
FDH	Lyophilized	5400 U/L	102 U/L
Formate	Sodium salt	0-8.89 mmol/L*** (0-400 mg/L)	0-0.14 mmol/L

*Combined as commercial reagent
**Combined into one working solution
***Based on amount added using acetonitrile pretreatment

Results

Figure 1 shows the change in absorbance over time following the addition of FDH to the reaction mixture. The reaction was complete in 7 min. The change in absorbance for the reaction product at 500 nm was < 1 mAu for all concentrations from 10 to 55 min after initiation of the reaction with FDH. For both direct and acetonitrile pretreatment procedures, a linear concentration-response relationship was obtained (0 to 400 mg/L, $r > .998$) and is illustrated in Figure 2. The slope of calibration relationship was reproducible from assay to assay: for the direct procedure, 2.42 ± 0.23 mAu/mg/L ($n = 12$ curves over five months) and for the acetonitrile pretreatment, 5.30 ± 0.14 mAu mg/L ($n = 5$ curves over three months).

Patient samples containing formate and supplemented with additional formate are shown in Table II. Recovery averaged $98.7 \pm 10.2\%$ in these 10 patient samples. Added concentrations of ethanol, methanol, acetone, ethylene glycol, lactate, acetaldehyde, formaldehyde, and acetonitrile did not interfere at the concentrations tested. At the 140-mg/L formate concentration, none of these added chemicals produced a deviation outside the range of 138 to 147 mg/L. At levels greater than 5000 mg/L for ethanol and methanol, slight positive biases began to be evident. In Table III, the effect of acetonitrile pretreatment vs. direct addition on absorbance values is seen for 15 hemolyzed.

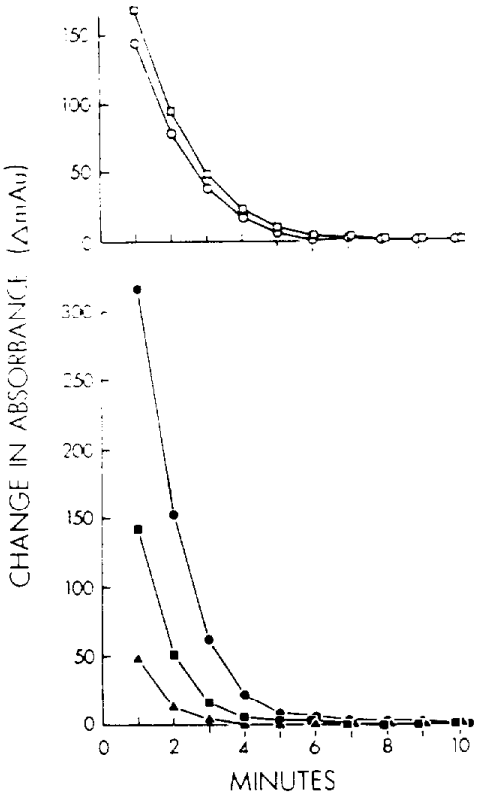

Figure 1. The change in absorbance (mAu) vs. time in the reaction cuvette after adding FDH. Samples were 100 μL of acetonitrile pretreated serum at 10 (▲), 50 (■), and 125 (●) mg/L, and 20 μL of serum (direct) at 330 (○) and 400 (□) mg/L

icteric, and lipemic samples. Though equal serum volumes were added for either pretreated or direct procedures, the acetonitrile pretreated samples gave lower values.

Intraassay and interassay precision data are given in Table IV. Least squares correlation of the enzyme method with acetonitrile pretreatment (y) against the gas chromatographic data (x) showed that slope = 0.980, intercept = 1.32 mg/L, correlation coefficient = 0.9944, and n = seven pairs of observations (Table V).

When compared to freshly prepared reagents, there were no differences in the slopes and intercepts of the calibration curves for buffered NAD-diaphorase stored at 0° to 8°C up to 19 weeks and for buffered NAD-diaporase-INT tested at six weeks. Formate concentrations were stable in serum when stored at −20°C for up to six months.

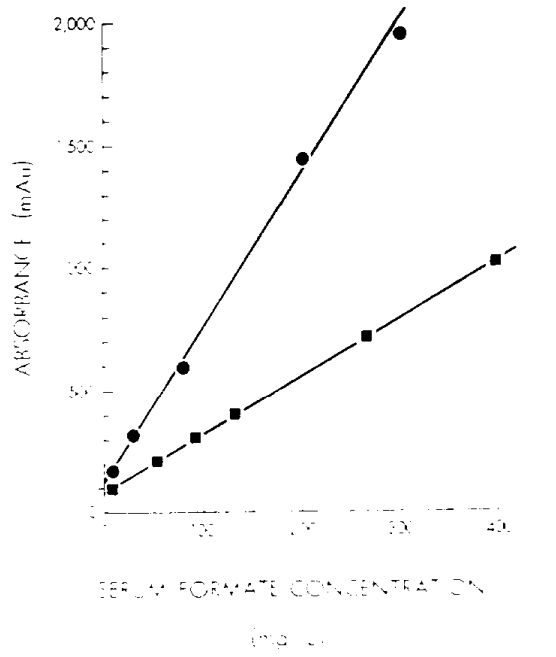

Figure 2. Calibration response for direct (■) and acetonitrile pretreatment (●) procedures.

Table II. Recovery Studies from Patient Samples

Serum Formate Concentrations (mg/L)				
Initial	Supplemental	Target	Recovered	Recovery (%)
3	+13	16	18	113
9	+13	22	22	100
20	+13	33	33	100
230	+13	243	246	101
237	+13	250	293	105
721	+13	734	737	100
9	+66	75	61	81
20	+66	86	70	81
230	+66	296	295	100
237	+66	303	321	106
			Mean	98.7
			SD	10.2

Discussion

Colorimetric chemical techniques based on the reduction of formate to formaldehyde are not always sensitive to concentrations of formate after overdose (<1500 mg/L), and many have poor recoveries. Isotachophoresis methodology is sensitive and has been used clinically (8) but the unfamiliar technology

Table III. Absorbance Values of Sera with Abnormal Appearance Using Direct Addition or Acetonitrile Pretreatment*

Sera Appearance	Absorbance (mAu)	
	Direct (20 μL)	Acetonitrile Pretreatment (40 μL)
Clear	83-92	49-68
Hemolyzed	211	96
	152	102
	113	64
	90	59
	67	51
Icteric	141	123
	69	64
	76	61
	94	63
	78	56
Lipemic	126	46
	143	52
	166	58
	106	53
	91	54

*Equal milliliters of serum acetonitrile were used as supernatant pretreated 20 μL of serum.

Table IV. Precision Studies

n	Concentration (mg/L)	Mean	SD	CV (%)
Within-run precision				
11	5	6.5	1.34	21
11	10	12.4	1.20	10
5	15	17.5	1.59	9
5	25	25.3	1.16	4
6	140	143.2	1.00	1
Between-run precision				
12	25	26.7	2.56	10
13	140	142.8	6.28	4

Table V. Formate Concentrations in mg/L Determined by Two Methods*

Patient Samples	Enzymatic	Gas Chromatographic**
A	263	267
B	117	118
C	78	82
D	24	38
E	27	7
F	5	6
G	5	3

*Intercept = 2.105, slope = 0.9675, and correlation = 0.9944.

**Commercially performed.

has limited widespread use in clinical laboratories. More commonly, formate can be determined using headspace gas chromatography after derivatization (15,16). While equally sensitive, such methods need a multi-step process requiring chromatographic expertise, partially dedicated gas chromatographic equipment, and more time than enzymic methods when total time for processing standards, controls, and samples is considered. Enzymic methods are based on the oxidation of formate to carbon dioxide by formate dehydrogenase with the production of reduced NAD (NADH). The NADH itself can be measured spectrophotometrically as is done in a procedure for urine (17) or by oxidizing NADH to NAD with diaphorase in a coupled reaction that reduces resazurin to resorufin, a fluorescent compound (13,14). Methods measuring only NADH production have a lesser calibration-response relationship (mAu · mg⁻¹ L) due to the lesser molar absorptivity of NADH, and suffer from interferences from turbidity or the appearance of the sample at 340 nm requiring decolorization. Fluorometric endpoint methods require the use of a fluorometer which is not as commonplace as a single-beam spectrophotometer. These methods also suffer from a limited dynamic range, poor calibration response (<0.5 response units · mg⁻¹ L) and non-linear calibration curves (13,14,18). In addition, between-sample variation in fluorescence prohibits accurate blanking, so standard addition methods were applied (18). Fluorescent interferences noted in glassware (14) were not seen with either glass or plastic tubes in the colorimetric assay described here.

In this procedure, the substitution of INT (molar absorptivity = 13,750 A · mol⁻¹ L) for resazurin and the precipitation of serum proteins with acetonitrile, eliminates some of the problems in previous enzymic procedures. The dynamic range was increased by greater than 2- to 10-fold over the modified (18) and original procedures (13,14). The calibration curve was linear rather than curvilinear over the 0 to 400 mg · L⁻¹ range. The modified fluorescence assay (18) was insufficient, in that there are only 0.4 fluorescence units (instrument resolution = 1 unit) · mg⁻¹ L. The greater calibration response of the authors' colorimetric assay (5 mAu · mg⁻¹ L) was due to the high molar absorptivity of INT and the fact that acetonitrile pretreatment allowed greater amounts of serum to be added into the reaction without proportionately increasing background absorbance. The original fluorescent procedure also used protein precipitation, but with $ZnSO_4$, NaOH (13).

Formate concentrations during methanol overdosage have been reported up to 930 mg · L⁻¹ depending on the sample timing. Endogenous concentrations have varied by report from 0 to 63 mg · L⁻¹ (8,11,15,19). However, the usual upper limit is considered to be 12 mg · L⁻¹. The limit of detection of the authors' assay is determined by procedural imprecision and non-specific absorbance. Table IV shows that concentrations less than 10 mg · L⁻¹ are imprecise, though qualitatively resolved from zero. High absorbances for "blank" serum reflect, in part, endogenous formate (regression of precision data indicate endogenous formate concentration of 4 mg/L in the serum used for making standards) and also non-specific absorbance. Dividing the blank serum absorbances or zero intercepts by the slope indicates that the limit of detection for quantitative work is 12 mg/L. Use of the method, as with the other formate methods, is not recommended for pharmacokinetic or comparison studies in the endogenous range (< 12 mg/L). Comparable results were obtained when compared to a commercial GC assay. External validation of the accuracy of enzymic assays has not previously been demonstrated.

Reagents (NAD, diaphorase, INT, buffer) can be prepared as one solution and stored conveniently at 2° to 8°C for six weeks. NAD and INT are present in 10- to 14-fold excess of amounts required for oxidation of the highest standard (400 mg/L). NADH is recycled in the coupled reaction and will not be depleted. No reversal of the enzymatic process was noted, when demonstrated by the stability of the final reduced chromagen. Concentrations of formate three times the highest standard would be required to exceed the specific activity of FDH, which is stable when lyophilized and stored at −70°C. Convenient pre-aliquoted portions (about 5 to 15 mg) can be stored and used for each batch of analyses. FDH will lose activity within hours after rehydration at room temperature.

The FDH-diaphorase reaction is specific for formate (13). The authors confirmed this for likely low molecular weight compounds that might also be present in these sera at concentrations exceeding or equal to those encountered clinically. Sigma analyzes each lot of FDH for contaminating dehydrogenases. Five different lot numbers were used, and only one had non-specific dehydrogenase activity. Since ethanol generated NADH with this lot, it was assumed to be an alcohol dehydrogenase. Because this could be a potentially significant interference, each lot should be checked and certified by Sigma. Some interference is due to abnormal serum character, interfering only at low concentrations. Though the degree of lipemia, bilirubinemia, and hemolysis was not quantified in this study, none of the lipemic, one of the icteric, and two of the hemolyzed sera produced high absorbances. While probably not a major problem at toxicologic concentrations of formate, duplicate blanks without FDH may help correct for the non-specificity.

Only a few reagents (sample, FDH, and buffered INT-NADH-diaphorase) are required. The analysis can be performed conveniently on a common single beam spectrophotometer. Reagents can be ready to use or deep frozen and thawed. The assay is currently being used to study methanol, formaldehyde, ethanol, and ethylene glycol overdoses.

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