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CHARACTERIZATION OF THE EFFECT OF METHYLCHLOROFORM INHALATION ON HEPATIC DRUG METABOLISM IN THE RAT

BY

ARNOLD MERWIN OLSHAN

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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ABSTRACT TITLE

EFFECT OF METHYLCHLOROFORM ON DRUG METABOLISM

ABSTRACT

Olshan, Arnold Merwin. M.S., University of Rhode Island, August 1969. Characterization of the Effect of Methylchloroform Inhalation on Hepatic Drug Metabolism in the Rat. Major Professor: Dr. George C. Fuller.

Male rats were exposed to methylchloroform (1,1,1-trichloroethane) vapors (concentration about 2500 PPM) for 24 hours and the effects of this treatment on drug responses and hepatic drug metabolism studied 24 hours later to determine the effect of methylchloroform on inducible hepatic drug-metabolizing enzymes.

Exposure to methylchloroform vapor significantly decreased the sleeping time of the rat in response to hexobarbital (120 mg/kg, i.p.) and zoxazolamine (80 mg/kg, i.p.).

Rat blood and liver methylchloroform content measured immediately following exposure suggested that the effects of methylchloroform were caused by a systemic action.

Experiments with 9000 x G supernatants indicated that the metabolism of hexobarbital was significantly increased following exposure to methylchloroform.

Significantly increased N-demethylation of aminopyrine, accompanied by increased amounts of microsomal CO-binding pigment

(cytochrome P-450) and microsomal NADPH-cytochrome c reductase were observed in in vitro experiments using hepatic microsomal fractions from rats exposed to methylchloroform. The spectral properties of the CO-binding pigment in animals exposed to methylchloroform did not differ from controls.

Pretreatment with actinomycin D or cycloheximide prevented the decrease in the hexobarbital sleeping time in rats exposed to methylchloroform. Further studies demonstrated that pretreatment with cycloheximide prevented the increase in metabolism of aminopyrine by rats exposed to methylchloroform.

These observations suggest that the increased drug-metabolizing activity in the exposed rats is due to an accelerated synthesis of the hepatic drug-metabolizing system. The inducing effects of methylchloroform appear to be related to those produced by phenobarbital.

MASTER OF SCIENCE THESIS

OF

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UNIVERSITY OF RHODE ISLAND

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I. INTRODUCTION

Humans may be exposed to small concentrations of methylchloroform (1,1,1-trichloroethane) vapors for extended periods of time because of its wide use as an organic solvent and degreaser. Knowledge of the possible mechanisms of toxic activity of methylchloroform would facilitate the development of a method of detecting early signs of toxicity of this liquid.

An increase in activity which appears to represent an increased concentration of enzyme protein is referred to as "enzyme induction." Induction of hepatic drug-metabolizing enzymes leads to accelerated biotransformations of drugs in vivo therefore altering the duration and intensity of drug actions in animals and man (Conney, 1967).

Studies by Gillette (1963) and Booth and Gillette (1962) have shown that foreign compounds in rats may enhance the activity of drug-metabolizing enzymes by at least three different mechanisms: one evoked by anabolic steroids, a second by polycyclic hydrocarbons, and a third by phenobarbital.

These three types of inducers stimulate varied pathways of drug metabolism by liver microsomes. This research was conducted to determine if methylchloroform is an inducer of hepatic drug-metabolizing enzymes and to identify methylchloroform with one

of the above three types of inducers if the above hypothesis proved correct.

The following parameters of hepatic drug metabolism were investigated: in vivo studies of hexobarbital and zoxazolamine sleeping time; in vitro metabolic studies of zoxazolamine hydroxylase, hexobarbital oxidase, aminopyrine demethylase, NADPH cytochrome c reductase, and CO-binding pigment; and studies of liver weight and microsomal protein. Inhibitors of protein synthesis (actinomycin D and cycloheximide) were used as tools to block the effect of methylchloroform on hepatic drug metabolism and thereby establish that an induction process does occur in response to methylchloroform exposure.

II. LITERATURE SURVEY

Methylchloroform

Description

Methylchloroform is a chlorinated hydrocarbon. It is a highly volatile, colorless liquid with a chloroform type odor (Browning, 1965).

Metabolism

Methylchloroform is readily absorbed through the lungs (Williams, 1959). Studies by Hake et al. (1960) showed that methylchloroform was very stable in the body, and while a large part of an intravenous dose was excreted unchanged by the lungs, a very small part was metabolized to chloroethanol and excreted in the urine as the glucuronate.

Effects in Animals

The primary toxic effect of methylchloroform is central nervous system depression producing anesthesia. Lethal dosage causes central nervous system depression resulting in respiratory paralysis (Adams et al., 1959). At the anesthetic level methylchloroform sensitizes the heart to epinephrine causing an idioventricular rhythm (Rennick et al., 1949). With chronic exposure (500 PPM, 7 hours a day, 5 days a week for 6 months) no evidence of impairment of growth or health was observed in rats, rabbits, guinea pigs, and monkeys. In another study at 2000 PPM for 3 months, the only animal species in

which organic injury was observed was the female guinea pig. These organic lesions included slight inflammation of the lungs and fatty changes in the liver (Torkelson et al., 1958). The lethal dose in rats by inhalation is 30,000 PPM for 6 minutes; 15,000 PPM for 1-1/2 hours; and 8000 PPM for 7 hours (Adams et al., 1950).

Effects in Man

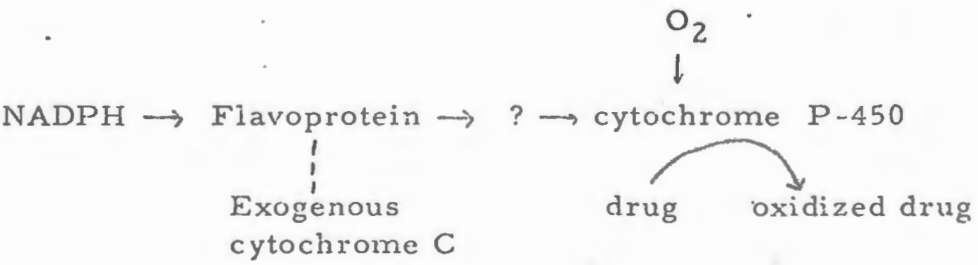
Stewart et al. (1961) have reported data obtained when seven human subjects were exposed to constantly increasing concentrations of methylchloroform up to 2650 PPM over a period of 15 minutes. At 1000-1100 PPM and 1900-2000 PPM mild eye irritation and throat irritation was noted, respectively, in 6 of the 7 subjects. At 2650 PPM 2 subjects were unable to stand and 3 subjects were very light-headed but able to stand. In another test at 910 PPM for 35 minutes, lightheadedness was experienced in one of two subjects. These subjective and physiological responses agreed with the results reported by Torkelson et al. (1958).

Stewart et al. (1961) also measured the blood and urine of subjects exposed to methylchloroform. Depending on length of exposure and concentration of methylchloroform, blood samples contained up to 10 PPM and urine samples up to 2 PPM at the termination of exposure. Thirty minutes after exposure only trace amounts (<1.0 PPM) of methylchloroform could be found in the blood or urine.

Previous investigations (Lal and Shah, 1968) have suggested a possible role of methylchloroform as an inducer of hepatic drug-metabolizing enzymes in rodents.

Oxidative Drug Metabolism

Early investigations have established that the enzymatic metabolism of foreign compounds is carried out by enzymes located in the microsomal fraction of the liver and are dependent on the generation of NADPH by the soluble fraction. Although the mechanism of drug oxidation by microsomes is not yet clear, Mason (1957) proposed that the oxidative reaction is coupled to a microsomal electron transport system operating between NADPH and oxygen. Siekevitz (1965) has reviewed the microsomal electron transport systems and suggests that the following pathway may be operative for the passage of electrons from NADPH to oxygen:



Cytochrome P-450 is the microsomal hemoprotein that is believed to function as the oxygen-activating enzyme and terminal oxidase for hydroxylating a number of drugs and steroids. Evidence for the existence of this cytochrome was first presented by Klingenberg (1958)

and Garfinkel (1948) and has since been extensively studied by Omura and Sato (1964). The hemoprotein can be characterized by its reactivity with carbon monoxide and ethyl isocyanide. The pigment has been termed cytochrome P-450 because in the reduced form reaction with carbon monoxide exhibits an absorption maximum at 450 mμ.

In 1965 Cooper et al. presented evidence for the participation of cytochrome P-450 in NADPH dependent microsomal systems responsible for the metabolism of many drugs. They showed that the liver microsomal oxidation of codeine, 4-monomethylamino-antipyrine, and acetanilide was inhibited by carbon monoxide and that this inhibition could be reversed by monochromatic light at 450 mμ or by oxygen. Kuntzman et al. (1968) implicated cytochrome P-450 as the terminal oxidase for steroid hydroxylation of testosterone by liver enzymes with his findings that the hydroxylation of testosterone by liver microsomes could be inhibited by carbon monoxide and that this inhibition was reversed by monochromatic light.

At the present time there is a question as to whether liver microsomes contain one or more different hemoproteins that function in hydroxylation reactions. Remmer and Merker (1965) and Sladek and Mannering (1966) have shown that phenobarbital or 3-methyl-cholanthrene increases the concentration of cytochrome P-450. Alvares et al. (1967) and Kuntzman et al. (1968) have shown that in 3-methylcholanthrene treated rats, carbon monoxide interacts with the

reduced microsomal hemoprotein to give an absorption maximum at 448 mμ instead of an absorption maximum at 450 mμ which is seen in the untreated and phenobarbital treated rats. Imai and Sato (1966) also presented evidence for two forms of cytochrome P-450 based on different spectral properties seen with phenobarbital or 3-methylcholanthrene treated rats when ethyl isocyanide was used as the ligand for the reduced hemoprotein. It is feasible that 3-methylcholanthrene or its metabolite binds to the hemoprotein, and this bound hemoprotein possesses spectral properties different from untreated or phenobarbital treated rats. Other possibilities are the induced synthesis of a spectrally different hemoprotein or in conversion of one form of hemoprotein to another form in the presence of the inducer. Kuntzman et al. (1968) and Alvares et al. (1967) have shown that pretreatment of rats with ethionine or actinomycin D prevented the peak shift to 448 mμ caused by 3-methylcholanthrene. In vivo administration of 3-methylcholanthrene to animals 2 hours prior to sacrifice or in vitro addition of 3-methylcholanthrene to microsomal suspension did not cause any spectral changes (Kuntzman et al., 1968). These results suggest the synthesis of a new hemoprotein but the true solution will have to wait for solubilization and purification of the hemoprotein(s) found in normal, phenobarbital and 3-methylcholanthrene animals.

Jick and Shuster (1966), Ernster and Orrenius (1965) and Remmer

and Merker (1965) have reported that treatment with phenobarbital increases liver microsomal NADPH cytochrome c reductase levels in the rat almost two fold. Von Der Decker and Hultin (1960) and Kato and Takayanaghi (1966) demonstrated that the administration of 3-methylcholanthrene had no effect on NADPH cytochrome c reductase. Thus, NADPH-cytochrome c reductase and P-450 are established as components of the oxidative drug-metabolizing system of liver microsomes and the activity of same should be evaluated in the presence of suspected induction of the drug-metabolizing enzymes.

Induction of Drug-Metabolizing Enzymes

At the present time at least 200 chemicals have been identified as inducers of drug-metabolizing enzymes (Conney, 1967). In studies where two different types of inducers (i.e., phenobarbital and 3-methylcholanthrene) were used together, liver microsomes were more active than studies where each was used alone or where two drugs of a similar type (i.e., 3-methylcholanthrene and 3,4-benzpyrene) were used together (Gillette, 1963). A similar in vitro study using ACTH and 3,4-benzpyrene (with zoxazolamine as the substrate) and phenobarbital and methyl testosterone (with mono-methyl-4-amino antipyrine as the substrate) showed a stimulation of drug metabolism more than maximum doses of 3,4-benzpyrene or phenobarbital could produce. This indicated a third possible type of inducer, the anabolic steroids.

These three general types of inducers stimulate varied pathways of metabolism by liver microsomes. There is a large overlap in the types of reactions and in specific pathways induced with individual drugs or chemicals. In general, 3-methylcholanthrene stimulates a more limited group of reactions than phenobarbital or the anabolic steroids.

Conney et al. (1960) and Kato and Takayanaghi (1966) have shown that 3-methylcholanthrene increases the metabolism of zoxazolamine in rats but has no effect on the in vitro metabolism of hexobarbital or aminopyrine, while phenobarbital significantly increased the metabolism of all three drugs. Anabolic steroids increase the metabolism of hexobarbital (Booth and Gillette, 1962); stimulate the N-demethylation of aminopyrine (Kato and Takayanaghi, 1966); but do not significantly alter zoxazolamine hydroxylase activity (Gillette, 1963; Kato and Takayanaghi, 1966).

Hexobarbital sleeping time is often used as an accurate indicator of microsomal drug metabolism. The duration of hexobarbital hypnosis in rats is significantly shortened by treatment with phenobarbital (Conney et al., 1960) and anabolic steroids (Booth and Gillette, 1962) but is not altered by 3-methylcholanthrene type drugs (Conney et al., 1960). The duration of zoxazolamine paralysis is significantly reduced by treatment of rats with phenobarbital or 3-methylcholanthrene (Conney et al., 1960), or in mice by anabolic steroids (Novick et al., 1966).

The time required for the various types of inducers to produce an effect differs. While phenobarbital produces stimulatory effects on enzyme activity in 1-4 days (Conney et al., 1960), polycyclic hydrocarbons take only 3 to 6 hours with maximum effects observed in 24 hours (Conney et al., 1956). Methyl testosterone induction is not apparent except after prolonged administration (weeks) (Booth and Gilette, 1962).

Phenobarbital, polycyclic hydrocarbons, and anabolic steroids may increase the levels of liver microsomal enzymes by stabilizing these enzymes as well as by accelerating the rate of synthesis. Kinetic studies with mouse tissue have shown that phenobarbital causes an increase in liver NADPH-cytochrome c reductase levels by increasing the rate of synthesis and by decreasing the rate of breakdown (Rubin et al., 1964).

While phenobarbital causes a 20-40% increase in microsomal protein per gram of liver (Conney et al., 1960), 3-methylcholanthrene (Conney et al., 1960) and the anabolic steroids (Booth and Gillette, 1962) cause no increase in microsomal protein per gram of liver. Phenobarbital and 3-methylcholanthrene administered to rats for 2 to 3 days increased the liver to body weight ratios 20 to 30% but prolonged treatment with anabolic steroids did not significantly change this ratio.

Electron microscopic examination of smooth surfaced

endoplasmic reticulum is a useful index of drug induced changes in liver microsomal enzyme levels. Phenobarbital causes proliferation of smooth surfaced endoplasmic reticulum in liver cells but has little effect on rough surfaced endoplasmic reticulum, while 3-methylcholanthrene has little or no effect on smooth surfaced endoplasmic reticulum (Remmer and Merker, 1965).

Inhibitors of Protein Synthesis

The induction of drug-metabolizing enzyme activity by phenobarbital or 3-methylcholanthrene is blocked by ethionine (Conney et al., 1956; Conney et al., 1960); puromycin (Gelboin and Blackburn, 1964; Orrenius et al., 1965); and actinomycin D (Gelboin and Blackburn, 1964; Orrenius et al., 1965).

These inhibitors block protein synthesis by various mechanisms. Ethionine appears to block protein synthesis by decreasing the ATP levels in the liver (Villa-Trevino et al., 1963); puromycin has been reported to block the transfer of soluble RNA-bound amino acid into microsomal protein (Yarmolinsky and De La Haba, 1956); and actinomycin D binds to DNA, and therefore may act by blocking the DNA directed synthesis of nuclear RNA required for protein synthesis (Reich et al., 1961). However, Reich (1964) has also reported that actinomycin D inhibits strand separation of helical DNA and suppresses DNA polymerase activity. Thus it is clear that actinomycin D

activity is exerted at the level of the nucleus.

Cycloheximide, another known inhibitor of protein synthesis, is reported to act on extranuclear components. Korner (1966) reported that the liver ribosomes of rats given cycloheximide by intraperitoneal injection incorporate less amino acids into protein than ribosomes from control rat liver. Their experiments showed that cycloheximide treated animals incorporated 95% less l-leucine-^{14}C into ribosomal proteins. Studies by Harris et al. (1968) show that a single dose of cycloheximide in rats inhibited the incorporation of uridine-^{3}H into nucleolar RNA with maximum inhibition at 24 hours.

III. EXPERIMENTAL

Animals

Male Sprague-Dawley derived rats obtained from Charles River Breeding Laboratories (Wilmington, Mass.), weighing between 60 and 100 gm. were used throughout the course of this study. The animals were maintained on commercial laboratory chow and water ad libitum. Rats were used not earlier than four days after being received from the supplier via commercial shippers or were used the following day when personally picked up at the source of supply. Rats were maintained in a room with controlled temperature and alternating 12 hour periods of light and darkness. All drugs were administered via the intraperitoneal route.

Materials

Analytical reagent grade chemicals or equivalent were used throughout this study. Co-factors (NADP, NADPH, glucose-6-phosphate, cytochrome c, and glucose-6-phosphate dehydrogenase) and crystalline bovine serum albumin were purchased from Calbiochem. The drugs used in this investigation were obtained from their respective manufacturers. The toluene used was the 25% fraction that remained after distillation.

Inhalation Chamber

The chamber consisted of a flat bottom cylindrical glass jar (12" x 18") placed horizontally on a wooden platform. The open end of the jar was closed by a wooden frame with a cork ring. The wooden frame was fitted with a removable circular wooden hatch (9/2" diameter) for the introduction of animals and a 4-hole rubber stopper (2" diameter). Two copper tubes reaching the end of the jar, a copper tube reaching only the front of the jar, and a thermometer were inserted through these holes.

Methylchloroform Exposure Protocol

The animals were placed into the inhalation chamber onto a perforated platform. The exposure atmosphere was formed by the passing of air at a rate of 0.2 liters/minute through a gas drying bottle containing methylchloroform and subsequent dilution with air at a flow rate of 10 liters air/minute. Equilibrium of the chamber atmosphere was achieved by introducing the air mixture through the long copper tubes described above with venting via the short copper tube. The desired flow rates were maintained by the use of flow meters (Hoke, Inc., Style 993). The animals were allowed free access to food and water for the entire period of exposure. At the end of the exposure, they were transferred to battery cages until used. The exposure protocol used for all studies relating to drug metabolism was a single 24 hour exposure with observations made 24 hours after the termination

of exposure. Those animals used for the assay of liver and blood were sacrificed immediately upon removal from the chamber.

Analytical Procedures:

Determination of Methylchloroform in the Exposure Chamber

An Aerograph Autoprep A-700 Gas Chromatograph (Variens, Inc., California) equipped with a thermal conductivity detector with a Speedomax X recorder was used to measure the concentration of methylchloroform in the exposure chamber. An 18% Hi Eff-3BP on 60/80 Chromosorb w A-10 column (neopentyl succinate on acid washed chromosorb) obtained from Applied Science (Philadelphia, Pa.) was used. The column temperature was set to about 73°C, injector temperature to about 130°C, and detector temperature to about 88°C. The filament was set at 200 ma. Helium with a gas flow of 60 ml/minute was used as the carrier gas. For the standard, 2 µl of pure methylchloroform was injected into the instrument at an attenuation of 16. For the sample, 20 ml of chamber air was injected into the chamber at an attenuation of 2. The areas under the standard and sample peaks were calculated and ppm were determined using the equivalent 1 mg/liter = 183 ppm (Browning, 1965).

Determination of Methylchloroform in the Blood

The blood was collected from rats as described below and extracted with 2 volumes of toluene. An Aerograph Hy Fi Model 600D-Linear temperature Programmer Model 326 equipped with an

Electron Capture Detector with a tritium source and a Honeywell Electronic 118 Recorder was used. A 20% GP 29A, ABS 60/70 column (20% carbowax 20M terminated with terephthalic acid groups on acid, alcohol, base washed diatomaceous earth) obtained from Analabs, Inc. (Hamden, Conn.) was used. Nitrogen was used as the carrier gas at a flow that permitted maximum separation. Since the determination was not linear, the following methylchloroform concentrations were used as standards: 0.1, 0.5, 1.0, 2.0, 4.0, 5.0, 6.0, 8.0, 10.0 ppm and a standard curve drawn. Two µl of all samples and standards were injected into the instrument. Peak heights were measured and the sample concentrations determined by extrapolation from a standard curve.

Determination of Sleeping Time

Duration of pharmacologic response of the depressant drugs employed in this study was measured using sleeping time as an end-point. Animals were weighed, marked, and placed into cages immediately prior to use. Hexobarbital solutions were prepared in distilled water with a small amount of 1N NaOH in a concentration which permitted the administration of 10 ml/kg. Zoxazolamine was prepared as a suspension in 0.5% methylcellulose in a concentration which also permitted the administration of 10 ml/kg. Sleeping time was taken as the length of time between the loss and regaining of the righting reflex.

Collection of Biological Samples

Blood. Methylchloroform-treated animals were sacrificed immediately upon removal from the exposure chamber (exposed 24 hrs. at atmospheric concentration of about 2500 ppm methylchloroform) by decapitation and the free-flowing blood collected in a beaker containing a few drops of 33% sodium citrate solution. The blood was frozen until assayed. Blood from control rats was collected in the same manner.

Liver. Immediately after decapitation and collection of blood (above), the livers were removed, homogenized in 1 volume of 0.1 M phosphate buffer (pH-7.4) and extracted with 2 volumes of toluene. These samples were stored in the freezer until assayed for methylchloroform. Control experiments established that no methylchloroform was lost during storage.

Preparation of Liver Microsomes

Animals were sacrificed by cervical dislocation, the abdomen opened and the livers perfused with 1.15% KCl solution via the hepatic portal vein. The livers were quickly excised, weighed, minced with scissors, and homogenized for 60 seconds in 2 volumes of ice cold 0.1 M phosphate buffer (pH 7.4) in a motor driven coaxial homogenizer. The homogenates were centrifuged for 30 minutes at 0° and 10,000 rpm (9000 x G ave.) in an International model B-60 preparative ultracentrifuge (rotor #874). The supernate was decanted and centrifuged at

30,000 rpm (78,000 x G ave.) for one hour to obtain the microsomal fraction. The microsomal pellet was washed and resuspended in cold 0.1 M phosphate buffer (pH 7.4) in a volume equivalent to that of the original liver. Aliquots of the microsomal suspension and 78,000 x G supernatants were frozen and stored at -10° C until assayed.

Preparation of 9000 x G Supernatants

Rats were sacrificed by cervical dislocation, and the livers homogenized as described above. The 9000 x G supernate fraction was taken after centrifugation at 0°C at 10,000 rpm in an International #874 rotor for 30 minutes and stored at -10°C until assayed.

Determination of Protein

9000 x G supernatant and microsomal protein were estimated by the method described by Lowry et al., (1951). About 165 mg. of liver equivalent for the 9000 x G supernatants and about 333 mg of liver equivalent for microsomes were placed into 50 ml centrifuge tubes containing enough 0.5 N NaOH for a final volume of 10.0 ml. The tubes were placed into a water bath and heated at 75°C until dissolution was complete. Standard solutions of freshly prepared crystalline bovine serum albumin in 0.5 N NaOH and a blank consisting of 0.5 N NaOH solution were carried through the entire procedure. When the tissue was completely dissolved, an 0.2 ml (9000 x G supernatants) or 0.4 ml (microsomes) aliquot was withdrawn and placed into

a second tube containing 1.0 ml of 0.5 N NaOH. Five ml of reagent A (1.0 ml of 2.7% potassium tartrate· $\frac{1}{2}$ H_2O, 1.0 ml of 1.0% $CuSO_4 \cdot 5H_2O$, and 100 ml of 2.0% Na_2CO_3) was added and the tubes mixed and allowed to stand at room temperature for 20 minutes. Then 0.5 ml of reagent B (commercial folin phenol reagent diluted to 1 N with distilled water) was added and the tubes mixed and allowed to stand at room temperature for 40 minutes before being read in a Beckman DB-G grating spectrophotometer at 500 mμ versus an 0.5 N NaOH blank.

Determination of In Vitro N-demethylation of Aminopyrine

The method of McMahon and Easton (1962) was employed to measure the formaldehyde formed during the in vitro N-demethylation of aminopyrine by liver fractions. A typical incubation mixture contained 1.0 ml of 9000 x G supernatant or 1.0 ml of 78,000 x G supernatant and 1.0 ml of microsomes (about 333 mg of liver equivalent), 10 μMoles of aminopyrine (substrate), 45 μMoles of semicarbazide, 30 μMoles of glucose-6-phosphate, 4 μMoles of NADP, 50 μMoles of $MgCl_2 \cdot 6H_2O$, 50 μMoles of nicotinamide, and 300 μMoles of 0.1 M phosphate buffer (pH 7.4) in a total volume of 5.0 ml in 25 ml erlenmyer flasks. Parallel incubation flasks containing 1.32 μMoles of formaldehyde (standard) in place of substrate and distilled water (blank) in place of substrate were carried through the entire procedure.

The flasks were incubated in a Dubnoff shaker at 37°C under air for 30 minutes. At the end of the incubation period, the flask contents

were poured into centrifuge tubes. Four ml of 10% zinc chloride (prepared with CO_2-free water) were added and the tubes mixed. Two ml of a saturated solution of barium hydroxide were added and the samples again mixed. The tubes were centrifuged for about 10 minutes and 5.0 ml aliquots of the clear supernates were transferred to clean centrifuge tubes containing 2.0 ml of double strength Nash reagent (0.04 M acetylacetone in 0.4 M ammonium acetate-0.1 M acetic acid). the contents were mixed and the tubes heated in a water bath at 60°C for 30 minutes. The colors were read against the blank using a Beckman DB-G grating spectrophotometer at 410 mμ.

Determination of In Vitro Metabolism of Hexobarbital

The method described by Cooper and Brodie (1953) was used to measure the disappearance of hexobarbital from incubation media. The basic incubation mixture and conditions as previously described were used with semicarbazide omitted and hexobarbital[1] as the substrate. After incubation for 30 minutes at 37°C in a Dubnoff shaker, the flask volumes were transferred to 50 ml centrifuge tubes containing 1.0 gm NaCl and 1.5 ml phosphate buffer (pH-5.5)[2].

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[1] A standard solution was prepared by dissolving 91.5 mg of hexobarbital in 200 ml of distilled water with 1.2 ml of 1 N NaOH. Incubation mixture used 1.0 ml (1.94 μMoles Hexobarbital Na) of the standard solution in total volume of 4.0 ml.

[2] Prepared by adding 8.5 ml of 0.119 Gm Na_2HPO_4 in 10.0 ml to 191.5 ml of 1.820 Gm KH_2PO_4 in 200 ml and washed with petroleum ether containing 1.5% isoamyl alcohol.

Thirty ml of washed petroleum ether[1] containing 1.5% isoamyl alcohol was added and the mixtures were shaken for 45 minutes and centrifuged for 10 minutes. Twenty ml aliquots of the solvent phase were transferred to clean tubes and shaken with 10.0 ml of phosphate buffer (pH-11)[2] for 10 minutes. The tubes were centrifuged and the petroleum ether was removed. The optical densities of the unmetabolized hexobarbital (aqueous phase) were determined at 245 mμ on a Beckman DB-G grating spectrophotometer. Two parallel incubation flasks were carried through the procedure, one containing 100 μgm hexobarbital sodium (standard) with boiled tissue and the other containing tissue but no hexobarbital sodium to serve as the blank.

Determination of In Vitro Metabolism of Zoxazolamine

The in vitro hydroxylation of zoxazolamine was measured by the method of Juchau et al., (1965). The basic incubation mixture and conditions as previously described were used with semicarbazide omitted and zoxazolamine[3] as the substrate. After incubation, 2.0 ml

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[1] Solvents were purified by washing with 1 N NaOH, 1 N HCl and two washings with distilled water. A technical grade of petroleum ether was adequate but a reagent grade of isoamyl alcohol was needed.

[2] Prepared by adding 8.5 Gm of NaOH to 500.0 ml of 0.8 M Na_2HPO_4 anhydrous.

[3] A standard solution was prepared by dissolving 50.0 mg of zoxazolamine in 50 ml of 0.1 M phosphate buffer (pH 7.4) with a small amount of 0.1 N HCl. Incubation mixture used 0.5 ml (3.0 μMoles zoxazolamine) of the standard solution in a total volume of 5.0 ml.

of the incubate was shaken with 25.0 ml of heptane containing 1.5% isoamyl alcohol and 0.5 ml of 1.0 N NaOH for 30 minutes and centrifuged for 5 minutes. Twenty ml of the heptane layer was pipetted into a second centrifuge tube containing 7.0 ml acetate buffer (pH 5.6), shaken for 5 minutes, and centrifuged for 5 minutes. Fifteen ml of the heptane layer was shaken for 15 minutes with 7.0 ml of 0.1 N HCl and centrifuged for 15 minutes. The optical density of the aqueous phase was read at 278 mμ against a 0.1 N HCl blank on a Beckman DB-G grating spectrophotometer. The amount of zoxazolamine metabolized was calculated as the difference in the amounts extracted from zero time (unincubated) and the incubated flasks.

Determination of Microsomal NADPH-Cytochrome c Reductase Activity

The system employed for the estimation of NADPH-cytochrome c reductase was a modification of that described by Ernster et al., (1962) and Dallner (1963). This assay is based on the determination of the rate of reduction of cytochrome c through measurement of the absorbance increase of the reduced cytochrome at 550 mμ.

Microsomal suspension containing 0.5 mg/ml of protein in 0.05 M phosphate buffer (pH 7.4) were employed for this assay. The assay system contained 0.67 μMoles NADPH, 0.33 μMoles cytochrome c, and 0.99 μMoles KCN in 0.05 M phosphate buffer (pH 7.4)

in a total volume of 3.0 ml. These reagents were placed in both the reference and sample cuvettes of a Beckman DB-G grating spectrophotometer equipped with a thermostatic cell compartment held at 37°C and a Beckman linear-log potentiometric recorder (Model 1005). A 0.1 ml aliquot (0.05 mg of protein) of microsomal suspension was introduced into the sample cuvette and mixed. The linear change in absorbance over a 5 minute period at 550 mμ was recorded. An extinction coefficient of 18.5×10^3 at 550 mμ for reduced minus oxidized cytochrome c (Margoliash, 1954) was used in the calculations. The results were expressed as μMoles of cytochrome c reduced per minutes per mg microsomal protein.

Determination of CO-Binding Pigment (P-450)

The system employed for the estimation of CO-binding pigment (cytochrome P-450) was similar to that described by Dallner (1963). The assay was conducted in a Cary Model 15 recording spectrophotometer.

To 6.0 ml microsomal suspension (prepared in 0.05 M phosphate buffer (pH 7.4) to contain 1 mg protein/ml) a few mg of Na dithionate were added to reduce the microsomal pigments. Three ml of microsomal suspension were placed into each of 2 cuvettes and the spectrum recorded from 500 to 400 mμ. Carbon monoxide was bubbled through the sample cuvette for a period of 1 minute. The spectrum was again scanned from 500 to 400 mμ and the absorbance

recorded. The absorbance at 500 mμ and at 450 mμ was taken from the recording and the content of the CO-binding pigment was expressed as the difference in absorbance between 450 and 500 mμ per mg of microsomal protein.

Statistical Methods

Student's t test (Dixon and Massey, 1957) was used to test for differences between means throughout this investigation. The formula employed is as follows:

$$t = \frac{X_1 - X_2}{s_p^2 \frac{(n_1 + n_2)}{n_1 n_2}}$$

where; $$s_p^2 = \frac{(n_1 - 1)s_1^2 \quad (n_2 - 1)s_2^2}{n_1 + n_2 - 2}$$

X_1 = control mean, X_2 = treatment mean

n_1 = control sample size, n_2 = treatment group sample size

s_1^2 = control variance, s_2^2 = treatment variance

Calculations were performed on an IBM 360 computer. The program was written in Fortran four language. The degrees of freedom were taken as $n_1 + n_2 - 2$. The levels of p were taken from appendix 5 (Dixon and Massey, 1957).

IV. RESULTS

In all experiments rats were exposed to a methylchloroform atmosphere (about 2500 ppm) for 24 hours.

Methylchloroform Concentration in the Exposure Chamber. The concentration of methylchloroform versus the two flow meters was determined several times throughout the investigation. The flow rates used for all experiments (0.2 liters/minute diluted with 10 liters air/minute) corresponded to a chamber atmosphere content of about 2500 ppm.

Methylchloroform Content of Rat Blood and Liver Following 24 Hour Exposure. Methylchloroform was found to be highly concentrated in the liver (127 ppm) in contrast to the blood (12.5 ppm) of rats exposed to the methylchloroform vapor (about 2500 ppm) for 24 hours (Table 1). Control experiments established that no methylchloroform was lost from blood samples collected and stored in a freezer before extraction with toluene. A peak corresponding to methylchloroform was observed in control samples although these animals had no known exposure to methylchloroform. This peak corresponds to instrument sensitivity.

All of the following data were obtained 24 hours after termination of a 24 hour period of exposure to the methylchloroform atmosphere.

TABLE 1

ANALYSIS OF METHYLCHLOROFORM IN THE BLOOD AND LIVER OF MALE RATS

	µg/gm[1]	mµMoles/gm
Liver		
Controls (3)	< 0.1	< 0.7
Treated[2] (3)	127.3 ± 8.7[3]	954.3 ± 65.1
	µg/ml	mµMoles/gm
Blood		
Controls (7)	< 0.1	< 0.7
Treated (8)	12.5 ± 2.3	93.7 ± 17.2

[1] Ug/gm were determined by gas chromatography using an Aerograph Hi Fi Chromatograph Model 600 D.

[2] Treated animals exposed to atmospheric concentrations of about 2500 ppm methylchloroform for 24 hours.

[3] Results are expressed as mean values ± standard error. Number of samples is in parentheses.

Effect of Methylchloroform on Hexobarbital Sleeping Time and Hexobarbital Oxidase

Hexobarbital sleeping time was significantly ($p < 0.001$) decreased (70%) in rats exposed to methylchloroform vapors (Table 2A). In other experiments, decreases ranging from 45-80% were observed. Significant ($p < 0.001$) increases of 30% in hexobarbital oxidase were observed in hepatic 9000 x G supernate of rats exposed to methylchloroform (Table 2B). These results are in agreement with experiments performed in mice (Lal and Shah, 1968).

Effect of Methylchloroform on Zoxazolamine Sleeping Time and Zoxazolamine Hydroxylase Activity

Zoxazolamine sleeping time was significantly ($p < 0.01$) decreased by 55 - 60% in rats exposed to methylchloroform (Table 3A). This was accompanied by a 20% increase ($p \leq 0.05$) in in vitro microsomal zoxazolamine hydroxylase activity (Table 3B).

Effects of Methylchloroform on Several Hepatic Microsomal Parameters of Drug Metabolism

The liver/body weight ratio was significantly ($p < 0.05$) increased by 29%. The trend in the experiments was for an increase of 20 - 30% in the ratio, but an increase was demonstrated in only half the experiments. As far as microsomal protein per weight of liver, this ratio never increased.

TABLE 2A

EFFECT OF METHYLCHLOROFORM ON HEXOBARBITAL SLEEPING TIME IN THE MALE RAT

	Sleeping time[1][2] (min. ± S.E.)
Controls	90.8 ± 4.9
Methylchloroform[3]	26.2 ± 3.0 *

1 Sleeping time taken as duration of loss of the righting reflex. (Hexobarbital Na 120 mg/kg).
2 Results are expressed as the mean values obtained from 11 animals ± standard error.
3 Methylchloroform animals exposed to atmospheric concentrations of about 2500 ppm methylchloroform for 24 hours.
* $P < 0.001$.

TABLE 2B

EFFECT OF METHYLCHLOROFORM ON THE METABOLISM OF HEXOBARBITAL IN RAT LIVER 9000 X G SUPERNATANTS

	µMoles/gm liver/30 min. ± S. E.[1]
Controls	2.541 ± 0.061
Methylchloroform[2]	3.365 ± 0.096*

1 Results are expressed as the mean values obtained from 10 animals ± standard error.
2 Methylchloroform animals exposed to atmospheric concentrations of about 2500 ppm methylchloroform for 24 hours.
* $P < 0.001$.

TABLE 3A

EFFECT OF METHYLCHLOROFORM EXPOSURE ON ZOXAZOLAMINE SLEEPING TIME IN THE MALE RAT

	Sleeping time [1] (min. ± S. E.)[2]	
	Control	MC[3]
Expt #1	124 ± 20.5 (7)	56 ± 6.8* (6)
Expt #2	106 ± 17.5 (7)	42 ± 5.5* (7)

[1] Sleeping time taken as duration of loss of righting reflex. (Zoxazolamine 80 mg/kg).
[2] Results are expressed as the mean values ± standard error with size in parentheses.
[3] MC = methylchloroform animals exposed to atmospheric concentrations of about 2500 ppm methylchloroform for 24 hours.
* $P < 0.01$

TABLE 3B

EFFECT OF METHYLCHLOROFORM ON ZOXAZOLAMINE HYDROXYLASE ACTIVITY IN THE MALE RAT

	mμMoles/mg microsomal protein/30 min. ± S.E.[1]	
	Control	MC[2]
Expt #1	210.9 ± 13.2	256.1 ± 14.4*
Expt #2	177.8 ± 2.7	216.6 ± 12.5*

[1] Results are expressed as the mean values obtained from 5 animals ± standard error.
[2] MC = methylchloroform animals exposed to atmospheric concentrations of about 2500 ppm methylchloroform for 24 hours.
* $P \leq 0.05$.

Further examination of Table 4 indicated the typical significant ($p < 0.001$) increases in CO-binding pigment (P-450) and NADPH-cytochrome c reductase activity by 40 and 34%, respectively. Spectral peaks of P-450 for both control and methylchloroform treated samples were the same, at about 451 mμ.

A 62% significant ($p < 0.001$) increase in demethylation of aminopyrine in the same group of rats was also noted. In other experiments 40 - 80% increases ($p < 0.001$) in the metabolism of aminopyrine were observed.

Effects of Cycloheximide on Various Parameters of Hepatic Drug Metabolism in the Male Rat

Cycloheximide, an extra-nuclear protein inhibitor, was used to prevent the decrease in hexobarbital sleeping time and increase in in vitro metabolism of aminopyrine. The data in Table 5 indicate that the significant decreases in hexobarbital sleeping time and significant increases in demethylation of aminopyrine caused by methylchloroform treatment, can be blocked by pretreatment with cycloheximide.

Data for liver weight and supernatant protein are also presented in Table 5. The methylchloroform plus cycloheximide group was not different from the methylchloroform plus saline group as far as weight of liver per weight of rat ratios but was significantly decreased ($P < 0.001$) in supernatant protein per gram of liver.

TABLE 4

EFFECTS OF METHYLCHLOROFORM ON SEVERAL HEPATIC MICROSOMAL CONSTITUENTS IN THE MALE RAT

	Wt. Liver per Body Wt. ratio (mg/gm ± S.E.)[1]	Microsomal protein per wt. liver ratio (mg/gm ± S.E.)	CO-binding Pigment (P-450) ($A_{450-500}$/mg/ml protein ± S.E.)	NADPH-cytochrome c reductase (μM/mg protein/min ± S.E.)	Demethylase (aminopyrine) (mμMoles HCHO[2] mg protein) ± S.E.
Control	53.4 ± 3.9	15.2 ± 1.2	0.053 ± 0.003	0.333 ± 0.018	176.6 ± 5.5
MC[3]	68.7 ± 3.3	15.4 ± 1.0	0.074 ± 0.003	0.447 ± 0.012	286.5 ± 17.5
"p" value	<0.05	>0.5	<0.001	<0.001	<0.001

[1] Results are expressed as mean values from 6 animals ± standard error.
[2] HCHO = formaldehyde.
[3] MC = Methylchloroform animals exposed to atmospheric concentrations of about 2500 ppm methylchloroform for 24 hours.

TABLE 5

EFFECTS OF CYCLOHEXIMIDE ON VARIOUS PARAMETERS IN THE MALE RAT

Parameter	liver wt/wt of rat ratio (mg/gm liver ± S.E.)[1]	9000 x G supernatant protein (mg/gm liver ± S.E.)	Hexobarbital Sleeping Time[2] (min ± S.E.)	Amino-pyrine Metabolism (mμMoles HCHO[3]/mg protein/ 30 min. ± S.E.)
Group				
Control & saline	41.8 ± 0.7	95.6 ± 2.4	78.3 ± 7.7	36.8 ± 2.2
MC[4] & saline	52.9 ± 0.8	104.4 ± 3.5	14.9 ± 0.9	64.6 ± 7.0
Control & cyclo[5]	47.7 ± 1.8	88.5 ± 2.1	121.5 ± 4.1	23.5 ± 2.2
MC & cyclo	50.0 ± 1.4	84.8 ± 2.5	87.1 ± 9.7	24.8 ± 2.6

1 Results are expressed as mean values of six rats ± standard error.
2 Sleeping time taken as duration of loss of righting reflex. (Hexobarbital Na 120 mg/kg.)
3 HCHO = formaldehyde.
4 MC = methylchloroform animals exposed to atmospheric concentrations of about 2500 ppm methylchloroform for 24 hours.
5 Cyclo = cycloheximide (2.5 mg/kg) half hour before MC treatment.

Effect of Actinomycin D on Hexobarbital Sleeping Time in the Male Rat

Treatment with actinomycin D (protein inhibitor at the nuclear level) at a dose of 40 µg/kg 30 min. before methylchloroform treatment and 40 µg/kg 12 hours later did not prevent the significant decrease in hexobarbital sleeping time caused by methylchloroform (Table 6A).

A dose of actinomycin D 80 µg/kg 30 min. before methylchloroform treatment and 40 µg/kg 12 hours later caused a significant inhibition ($p < 0.01$) of the methylchloroform induced decrease in hexobarbital sleeping time (Table 6B).

TABLE 6A

EFFECT OF ACTINOMYCIN D ON HEXOBARBITAL SLEEPING TIME IN THE MALE RAT

Group	Sleeping time[1] (min. ± S.E.)[2]
Control	112.5 ± 8.7 (6)[3]
MC[4]	44.8 ± 6.0 (6)
Control & actino[5]	111.5 ± 12.2 (6)
MC & actino	51.7 ± 8.3 (6)

[1] Sleeping time taken as duration of loss of righting reflex.
[2] Results are expressed as the mean values ± standard error.
[3] Animals per group.
[4] MC = methylchloroform animals exposed to atmospheric concentrations of about 2500 ppm methylchloroform for 24 hours.
[5] Actino = actinomycin D 40 µg/kg 30 min. before MC treatment and 40 µg/kg 12 hours later.

TABLE 6B

EFFECT OF ACTINOMYCIN D ON HEXOBARBITAL SLEEPING TIME IN THE MALE RAT

Group	Sleeping time[1] (min. ± S. E.)[2]
Control	116 ± 5.7 (7)[3]
MC[4]	48 ± 2.1 (8)
Control & actino[5]	118 ± 4.2 (8)
MC & actino	72 ± 7.1 (10)

[1] Sleeping time taken as duration of loss of righting reflex.
[2] Results are expressed as the mean values ± standard error.
[3] Animals per group.
[4] MC = methylchloroform animals exposed to atmospheric concentrations of about 2500 ppm methylchloroform for 24 hours.
[5] Actino = actinomycin D 80 µg/kg 30 min. before MC treatment and 40 µg/kg 12 hours later.

V. DISCUSSION

This investigation indicated that hepatic drug-metabolizing enzymes in rats are stimulated by exposure to methylchloroform vapor. Methylchloroform was detected and measured in the liver and blood of exposed rats thus providing justification for a hypothesis that methylchloroform exerts a systemic effect. Studies by Stewart et al. (1961) in humans confirmed that methylchloroform blood concentrations are related to exposure.

Exposure to methylchloroform vapor significantly shortened hexobarbital and zoxazolamine sleeping time as do inducers of the phenobarbital type (Conney et al., 1960) and anabolic steroid type (Booth and Gillette, 1962; Novick et al., 1966). Inducers of the 3-methylcholanthrene type only reduce zoxazolamine sleeping time (Conney et al., 1960).

In vitro parameters of drug metabolism (hexobarbital oxidase, aminopyrine N-demethylase, and zoxazolamine hydroxylase) were significantly increased by methylchloroform exposure. Phenobarbital has been reported to cause increases in the same parameters (Conney et al., 1960; Kato and Takayanaghi, 1966). Anabolic steroids significantly increase the in vitro metabolism of hexobarbital and aminopyrine (Booth and Gillette, 1962; Kato and Takayanaghi, 1966),

but do not significantly alter the hydroxylation of zoxazolamine (Gillette, 1963; Kato and Takayanghi, 1966). 3-Methylcholanthrene increases the metabolism of zoxazolamine, but not aminopyrine or hexobarbital (Conney et al., 1960; Kato and Takayanghi, 1966).

Methylchloroform significantly increased the levels of NADPH-cytochrome c reductase and the content of CO-binding pigment (P-450) and did not change the spectral properties of the hemoprotein (P-450) when compared to controls. Treatment of rats with phenobarbital significantly increases the activity of the liver microsomal NADPH-cytochrome c reductase (Jick and Shuster, 1960; Ernster and Orrenius, 1965) and the content of CO-binding pigment (Kuntzman et al., 1968) without changing the spectral properties of this pigment. Kato and Takayanaghi (1966) and Von Der Decken and Hultin (1960) have demonstrated that treatment with 3-methylcholanthrene had no effect on NADPH-cytochrome c reductase activity while Alvares et al. (1967) and Kuntzman et al. (1968) have shown increases in the content of CO-binding pigment, but with different spectral properties when compared to controls.

Inhibitors of protein synthesis have been demonstrated to block the increase in hepatic drug metabolism by known inducers. Pretreatment of animals with actinomycin D and cycloheximide prevented the induction of significant decreases in hexobarbital

sleeping time by methylchloroform. The increase in the metabolism of aminopyrine was also blocked by cycloheximide pretreatment. These data support the hypothesis that the increase in drug-metabolizing enzymes of animals exposed to methylchloroform represents an induction of more enzyme protein.

VI. SUMMARY AND CONCLUSIONS

Rats were exposed for 24 hours to an atmospheric concentration of about 2500 PPM methylchloroform and *in vitro* and *in vivo* parameters of drug metabolism were measured 24 hours after the termination of exposure.

(1) Methylchloroform content was 10-fold greater in the liver than the blood of exposed rats sacrificed immediately upon removal from the chamber. These findings suggest that methylchloroform acts systemically.

(2) Hexobarbital and zoxazolamine sleeping times were significantly decreased by exposure to methylchloroform vapor.

(3) *In vitro* metabolism of hexobarbital, zoxazolamine, aminopyrine, NADPH-cytochrome *c* reductase, and content of CO-binding pigment were significantly increased in animals exposed to methylchloroform.

(4) Actinomycin D and cycloheximide blocked the methylchloroform induced decrease in hexobarbital sleeping time. Cycloheximide blocked the methylchloroform induced increase in demethylation of aminopyrine. These data suggest that the increase in the drug-metabolizing enzymes of the animals exposed to methylchloroform was mediated through the synthesis of protein.

(5) The spectrum of enzymes induced by methylchloroform

appears to be closely correlated with the spectrum of enzymes induced by phenobarbital.

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