HEI HEALTH EFFECTS INSTITUTE

Determination of the Atherogenic Potential of Inhaled Carbon Monoxide

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Includes the Commentary of the Institute's Health Review Committee

Research Report Number 57
May 1993

HEI HEALTH EFFECTS INSTITUTE

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HEI Statement
HEALTH EFFECTS INSTITUTE

Synopsis of Research Report Number 57

Carbon Monoxide and Atherosclerosis

Background

Carbon monoxide is a ubiquitous air pollutant. It is found in cigarette smoke and emissions from motor vehicles, industrial processes, and poorly ventilated combustion sources. Despite reductions in ambient carbon monoxide concentrations during the last decade, 22 million people live in areas of the United States that exceed the National Ambient Air Quality Standard for carbon monoxide (9 parts per million [ppm] averaged over eight hours and 35 ppm averaged over one hour). Inhaling carbon monoxide is dangerous because the gas binds to hemoglobin in red blood cells to form carboxyhemoglobin. This displaces oxygen and reduces hemoglobin's ability to deliver oxygen to body tissues. If tissues are deprived of oxygen, transient or permanent damage can occur, especially in those organs that demand high oxygen delivery, such as the brain and heart. The lethal consequences of exposure to high concentrations of carbon monoxide are well known. There is also concern that exposure to low levels of carbon monoxide may produce adverse effects, especially on the cardiovascular system.

Carbon monoxide could manifest its toxic effects on the heart and blood vessels in two ways, either by causing acute, short-term effects on oxygen delivery or by contributing to the development of cardiovascular diseases such as atherosclerosis. Atherosclerosis is a progressive disease characterized by thickening of the arteries and a buildup of deposits of fat, cholesterol, cells, and connective tissue in the inner lining of the blood vessels. These deposits, called plaques, can partially or totally block the flow of blood through the artery. The known risk factors for atherosclerosis are family history of the disease, diet, high blood pressure, and cigarette smoking. (Mainstream and sidestream cigarette smoke have extremely high levels of carbon monoxide [400 to 1,000 ppm]). It is important to know whether chronic exposure to ambient levels of carbon monoxide is also a risk factor for developing atherosclerosis because this disease is the leading contributor to deaths by heart attack and stroke in the United States.

Approach

Birds are appropriate animal models for studying atherosclerosis because some species develop spontaneous atherosclerotic plaques that resemble those in humans. Dr. Arthur Penn and his colleagues previously had shown that the development of atherosclerotic plaques in cockerels (young roosters) was enhanced when the birds were exposed to cigarette smoke or treated with agents that stimulate cell proliferation, such as chemical carcinogens. In the study described here, Dr. Penn exposed cockerels to either air or defined levels of carbon monoxide (50 to 200 ppm) for 16 weeks and measured the size of the atherosclerotic plaques in the abdominal aorta. He also examined whether carbon monoxide promoted atherosclerosis when the birds were fed a low cholesterol (0.1%) diet and exposed to carbon monoxide (100 ppm) at the same time. Finally, he determined whether carbon monoxide exposure altered the development of preexisting plaques.

Results

In this study, none of the carbon monoxide exposures, either alone, or in combination with a low cholesterol diet, had an effect on the rate of development or the regression of atherosclerotic plaques in cockerels. Reliable blood carboxyhemoglobin measurements were not obtained. Consequently, there was no biomarker linking external carbon monoxide exposure concentrations and internal dose. Because birds and mammals differ in their uptake and elimination of carbon monoxide, it is possible that the actual carbon monoxide body burden was relatively minimal and not comparable to human exposures. Considering this uncertainty, the results of this study do not provide a definitive answer to the question of whether exposure to carbon monoxide is a risk factor for atherosclerosis. The evidence favoring such a role at ambient carbon monoxide levels is not strong; however, because of the importance of the problem, the issue warrants further evaluation.

This Statement is a summary, prepared by the Health Effects Institute (HEI) and approved by the Board of Directors, of a research project sponsored by HEI from 1986 to 1988. This study was conducted by Dr. Arthur Penn at New York University Medical Center. The complete report (Determination of the Atherogenic Potential of Inhaled Carbon Monoxide, Research Report Number 57) can be obtained from the Health Effects Institute, 141 Portland Street, Suite 7300, Cambridge, MA 02139 (Telephone: 617-621-0266). The Research Report contains both the detailed Investigator's Report and a Commentary on the study prepared by the Institute's Health Review Committee.
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Library of Congress Catalog No. for the HEI Research Report Series: WA 754 R432.

The paper in this publication meets the minimum standard requirements of the ANSI Standard Z39.48-1984 (Permanence of Paper) effective with Report Number 21, December 1988, and with Report Numbers 25, 26, 32, and 51 excepted. Reports 1 through 20, 25, 26, 32, and 51 are printed on acid-free coated paper.

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ABSTRACT

The effects of chronic exposure to moderate levels of carbon monoxide upon the augmentation of arteriosclerotic plaque development were investigated in a series of in vivo studies in the cockerel (young rooster). This animal model has been well characterized, especially regarding the role of environmental agents in exacerbating early stages of plaque development. Cockerels injected with subtumorigenic doses of carcinogens exhibit markedly accelerated development of aortic arteriosclerotic plaques. Inhalation of mainstream smoke from two packs of cigarettes (100 minutes/day for 16 weeks) causes small but statistically significant increases in plaque size. As is the case with many animal models of plaque development, raised fat-proliferative plaques also appear in these animals following cholesterol feeding.

Carbon monoxide is a ubiquitous pollutant in urban environments, where it is derived largely from mobile sources and cigarette smoke. Exposure to chronically elevated carbon monoxide levels has been implicated in a number of health-related problems. Whether such exposure plays a role in the development of arteriosclerosis has not been determined conclusively.

In the present study, three questions were posed:

1. Will inhaled carbon monoxide at levels of 50 to 200 parts per million (ppm)* (two hours/day for 16 weeks) be sufficient to augment arteriosclerotic plaque development in cockerels, in the absence of other plaque-promoting agents?
2. Will the inhalation of 100 ppm carbon monoxide (two hours/day for 16 weeks), concomitant with the feeding of low levels (0.1%) of cholesterol, yield larger plaques than those obtained with either of these agents administered alone?
3. Will inhalation of 100 ppm carbon monoxide (two hours/day for 11 or 22 weeks), by cockerels in whom plaques have already appeared, further augment plaque development?

Cockerels were exposed to carefully regulated levels of carbon monoxide in stainless-steel and Plexiglas dynamic exposure chambers. The volume percentage of plaques in the wall of the abdominal aorta of each exposed and control animal was determined by a point-counting method.

Chronic inhalation of carbon monoxide at levels as high as 200 ppm did not affect augmentation of arteriosclerotic plaque development. (In separate studies involving inhalation of 200 ppm carbon monoxide, carboxyhemoglobin levels 10 minutes after exposures ended were 11% to 12%.) When administered at the same time that plaque development was being promoted by cholesterol feeding, carbon monoxide had no further effect upon plaque development. When administered after either carcinogen-associated or diet-augmented increases in plaque size, carbon monoxide did not elicit further increases in plaque size. Thus, in this animal model, daily exposures to moderately high levels of carbon monoxide were without discernible effect upon arteriosclerotic plaque development.

INTRODUCTION

In the United States and western Europe, cardiovascular diseases are responsible for the deaths of more people than all forms of cancer combined (Silverberg and Lubera 1989). Both clinically and experimentally, the principal lesion associated with cardiovascular disease is the arteriosclerotic plaque. Plaques arise in the intimal region of the wall of medium to large arteries. The intimal region is bordered by a single layer of endothelium on the lumenal surface and by the multilayered media. The smooth muscle cell is the predominant cell type in both the media and in plaques. Proliferation of intimal smooth muscle cells is considered essential for the development of arteriosclerotic plaques. In contrast, medial smooth muscle cells generally exhibit very low levels of proliferation. In addition to smooth muscle

* A list of abbreviations appears at the end of the Investigator's Report.

This Investigator's Report is one part of the Health Effects Institute Research Report Number 57, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigator's Report may be addressed to Dr. Arthur Penn, Institute of Environmental Medicine, New York University Medical Center, 550 First Avenue, New York, NY 10016.

Although this document was produced with partial funding by the United States Environmental Protection Agency under assistance agreement 816285 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement should be inferred.

cells, plaques contain lesser but variable numbers of T-lymphocytes and macrophages, as well as collagen, elastin, glycosaminoglycans, and lipid deposits. In large plaques, a combination of lipids, dying cells, and cellular debris will often form a gruel-like core (atheroma). In advanced plaques, calcification can also be present. There is strong epidemiological evidence for an interaction of genetic factors and complex environmental mixtures (e.g., cigarette smoke and diesel exhaust) in the development of both clinically and experimentally significant cardiovascular disease. However, the contribution of individual pollutants to the development of this disease is not well understood.

Carbon monoxide (CO) is a common pollutant, especially in industrial societies. It has been estimated that more than 80% of the CO in urban air is derived from mobile sources. Tobacco smoke accounts for a large part of the remainder. Whether chronically elevated CO levels can play an important role in the development of arteriosclerosis is still not clear.

In June 1983, the Health Effects Institute (HEI) issued an RFA entitled "Cardiovascular and Other Health Effects of Carbon Monoxide." According to the Clean Air Act, it is not sufficient to demonstrate that ambient levels of a specific pollutant are without deleterious effects upon most members of the general population. The Clean Air Act mandates that air quality standards be set to protect susceptible groups in the population from airborne pollutants that might compromise their health. Thus, in its RFA for CO, HEI emphasized that " . . . identifying factors making individuals sensitive and quantifying their sensitivity is very important". The health effects resulting from acute CO exposures have been documented extensively. In cases in which this link hasn't been proven directly, it has been assumed that the major health effects of CO are due to the binding of CO to hemoglobin and the resulting diminution in oxygen delivery to affected tissues. In the August 1984 addendum to the 1979 U.S. Environmental Protection Agency Air Quality Criteria Document for CO, the Clean Air Scientific Advisory Committee concluded unanimously that, with regard to cardiovascular effects, the greater affinity for hemoglobin of CO than oxygen was the key mechanism of CO toxicity (U.S. Environmental Protection Agency 1984).

At the time that the RFA was issued, the difficulty of ascribing cardiovascular effects to chronic CO exposure was compounded by the scanty and conflicting evidence from animal studies. In some cases, high levels of CO had been used. In other cases, the treatment regimens involved combinations of CO inhalation along with dietary supplements of cholesterol (0.5% to 2.0%). Hellung-Larsen and associates (1968) reported that lactate dehydrogenase isozymes were altered and that more frequent and severe arteriosclerotic plaques appeared in the aortic arches of rabbits exposed for relatively short periods of time to 550 ppm CO. Kjeldsen and colleagues (1972) exposed rabbits to 180 ppm CO continuously for four weeks. They reported severe edema, subendothelial blisters, and plaque development. The plaque development was attributed to tissue hypoxia. Carboxyhemoglobin (COHb) levels of 15% were attained. In other animal studies, treatment regimens often involved combinations of CO inhalation and dietary cholesterol supplements. Davies and coworkers (1976) fed rabbits a diet supplemented with 2% cholesterol and exposed the animals to 150 ppm CO, four hours/day, for 70 days. Coronary artery atherosclerosis was significantly higher in CO-exposed animals than in cholesterol-fed, unexposed controls. However, 2% is a fairly high level of cholesterol, and the CO exposures resulted in blood COHb levels of 20%. Similar results were reported when monkeys (Webster et al. 1970) and pigeons (Turner et al. 1979) were treated to CO and cholesterol regimens. The results of these studies indicated that the severity of atherosclerosis is correlated both with the CO dose and the level of dietary cholesterol. However, in these cases as well, doses were relatively high. The potential atherogenic effects of combining exposures of animals to low-to-moderate doses of CO with low-to-moderate levels of dietary cholesterol have not been investigated adequately.

There have been numerous studies of CO levels in urban atmospheres. The National Ambient Air Quality Standard is 35 ppm for one hour (U.S. Environmental Protection Agency 1971). Most studies indicate that median urban CO levels are generally lower than 20 ppm. However, there have been a variety of studies showing that CO levels can climb to 50 ppm and above in specific urban locations or under specific driving conditions. For example, in unventilated pedestrian underpasses, mean CO ranges from 70 ppm to more than 100 ppm have been reported (Wright et al. 1975). In indoor ice skating and hockey rinks, high levels of CO are generated by the ice resurfacing equipment (Spengler et al. 1978). In some cases, ambient rink CO levels have exceeded the national standard by 300%. The greatest source of urban CO, by far, is generated by moving vehicles. Studies of CO levels at fixed street locations have shown that although values can be high, they are generally within the acceptable range (Reed and Trott 1971). However, one English study has indicated that the CO levels to which drivers within moving cars are routinely exposed may be a good deal higher than previously believed (Colwill and Hickman 1980). Average levels as high as 60 ppm were recorded in that study. Due to the delays often associated with automobile commuting in an urban environment, there is a good chance that every day a large part of the urban work force is exposed to elevated CO levels for one to two hours, depending on commuting time. In addition, toll takers at bridges and tunnels

can face elevated CO exposures for even longer periods of time. Thus, chronic exposure to moderately high CO levels is a reality for many people.

Increased inhalation of CO leads to elevated COHb levels. Impairment of physiologic function (e.g., decreased exercise efficiency, changes in cardiac and pulmonary function, headaches, drowsiness, and ultimately, coma and respiratory failure) (National Research Council 1977) correlates well with increased COHb levels. However, despite these and other related studies (Kurt et al. 1979), it is by no means clear whether chronic exposure to elevated CO levels plays a direct role in the exacerbation of cerebro- or cardiovascular disease. It is worth noting that at an international workshop on CO and cardiovascular disease held more than 10 years ago, the conclusion was reached that in otherwise healthy individuals (i.e., those not predisposed to cardiovascular disease) CO exposures leading to elevated COHb levels were probably without chronic consequences (Gori 1979). In those individuals already predisposed to cardiovascular disease, exposure to elevated CO levels would be expected to be particularly deleterious. Until now it has been very difficult to determine in a straightforward fashion whether chronic exposure to elevated CO can accelerate arteriosclerotic plaque development.

There is extensive evidence that avian species can serve as relatively inexpensive animal models for studying the effects of diet and other environmental factors upon the development of arteriosclerotic plaques. In turkeys, spontaneous aortic plaques appear within the first three months of life (Simpson and Harms 1968). Japanese quail have been employed as a model both for testing antiatherosclerosis drugs (Day et al. 1977) and for studying viral involvement in atherosclerosis (Pyrzak and Shih 1987). Spontaneous plaques were described over 30 years ago in pigeons (Clarkson et al. 1959). Subsequent identification of strains that are either susceptible to atherosclerosis or resistant to it (St. Clair et al. 1974) has led to a number of detailed studies of the effects of diet on plaque development. Chickens have been used as animal models of atherosclerosis even longer than pigeons. It was noted 50 years ago (Dauber and Katz 1942; Dauber 1944) that feeding cholesterol to chickens enhanced the development of atherosclerosis. A role for estrogens in inhibiting plaque development and in promoting plaque regression in cholesterol-fed chicks was described 10 years later (Pick et al. 1952), and the development of ulcerated atherosclerotic lesions in the cockerel was described 10 years after that (Pick et al. 1962). Arteriosclerotic plaques in the cockerel abdominal aorta are very similar to human fibromuscular coronary artery plaques (Moss and Benditt 1970), and these cockerel plaques respond to environmental agents that stimulate cell proliferation (Albert et al. 1977; Penn et al. 1981a,b; Penn and Snyder 1988). These agents include a variety of individual carcinogens, as well as cigarette smoke. The initial arteriosclerotic responses to some of these stimuli can be detected before eight weeks of age (Penn et al. 1980, 1981a). However, CO at levels comparable to those found in cigarette smoke does not stimulate plaque development (Penn et al. 1983). Other than size, there are no major differences, even ultrastructurally, between these experimentally manipulated cockerel plaques and quiescent spontaneous plaques (Batastini and Penn 1984). Finally, DNA from both cockerel (Penn et al. 1991) and human plaques (Penn et al. 1986) has transforming potential. Thus, similar molecular alterations have occurred in both sets of plaques.

The experiments we proposed addressed directly two of the stated objectives of the HEI RFA dated June 30, 1983: "Measurement of a variety of physiological parameters in healthy animals and in those with cardiovascular disease in response to CO" and "effects of long-term exposure to low or moderate levels of CO on atherosclerosis".

The end points we selected were the accelerated appearance and enhanced development of arteriosclerotic plaques. During the course of a previous study on the plaque-stimulating effects of mainstream cigarette smoke, we found that CO (50 ppm, 100 minutes/day for 16 weeks) elicited small but significant increases in COHb levels without stimulating plaque development in cockerels (Penn et al. 1983).

OBJECTIVES AND RATIONALE

There were three major objectives in this study:

1. To determine whether there is a level of CO (up to 200 ppm) which, with chronic exposure, will be sufficient to augment the development of arteriosclerotic plaques in the absence of other plaque-promoting agents;
2. To determine whether a moderately high level of CO (100 ppm) will promote plaque development when combined with a diet that is mildly elevated in cholesterol; and
3. To determine whether daily exposure to a subatherogenic concentration of CO will lead to enhanced development of preexisting arteriosclerotic plaques.

Unlike the exposure protocols employed in previous studies by other investigators, we proposed to employ chronic exposure to low-to-moderate CO levels for periods of up to 20 weeks in the presence and absence of low-to-moderate levels of dietary cholesterol or low levels of the carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA), which stimulates plaque development in cockerels at subtumorigenic doses (Penn et al. 1981a,b). Because the aortic plaques in the cockerel appear early in life, are very similar to fibrous human coronary artery plaques, and are very sensitive to environmental agents, we reasoned that this system should provide

valuable information on modulation of plaque development that might even be extrapolated ultimately to the human situation. This system would be especially valuable as a model for individuals who may be susceptible to exacerbation of cardiovascular disease by common environmental agents. By focusing upon relatively early phenomena of plaque development, e.g., smooth muscle cell proliferation and the resulting sharp increase in plaque size, we hoped to learn whether CO could exacerbate arteriosclerotic plaque development and, if so, to begin to identify the mechanisms involved.

We had already demonstrated that exposure of cockerels to 50 ppm CO for 100 minutes/day from 4 to 20 weeks of age does not by itself enhance development of arteriosclerotic plaques (Penn et al. 1983). This level of CO is relatively high by urban, if not experimental, standards. We expected to find that there are even higher concentrations of CO, that, by themselves, would not contribute appreciably to plaque development. Additionally, we anticipated that there is a level of CO that, although not atherogenic by itself, would exacerbate plaque development in animals fed a diet supplemented with a low level of cholesterol. Finally, we hoped to obtain evidence that chronic inhalation of CO acts to stimulate the further development of preexisting arteriosclerotic plaques.

MATERIALS AND METHODS

ANIMAL CARE

Four-week-old white leghorn cockerels (Kerr Hatcheries, Frenchtown, NJ) were distributed randomly into stainless-steel dog cages and quarantined for two weeks before exposures began. The cage dimensions were 33" w × 38" d × 30" h. There were never more than four cockerels per cage. The animals were kept on a 12-hour-light (6 am to 6 pm)/12-hour-dark cycle. The room temperature was kept at 22°C to 23°C at all times, with ambient relative humidity. Filtered air (single pass) was changed 12 times per hour. Animals were weighed weekly, and their health was monitored daily.

Cockerels were provided with water and food (Chick Starter Grower, Purina, St. Louis, MO) ad libitum, except for the time when they were in the exposure chambers. This specially formulated, vitamin-supplemented diet is designed for chickens during their first year of life. The levels of dietary components were as follows: crude protein (from corn, wheat, alfalfa, and soybean), greater than 21%; crude fat, predominantly from soybean oil, greater than 3%; crude fiber, less than 4%; ash, less than 7%; added minerals, less than 3.5%. Analyses were provided by the supplier. For the cholesterol-supplemented diets, chemically pure cholesterol, not derived from animal sources, was dissolved in a minimum amount of ethanol and thoroughly mixed with the feed by the supplier.

All animal handlers were trained and certified by the American Association for the Accreditation of Laboratory Animal Care. Housing of cockerels within the New York University Medical Center Institute of Environmental Medicine animal facilities was in accordance with applicable portions of the Animal Welfare Act (1966) (P.L. 89–544, as amended by P.L. 91–579, 1970, and 94–279, 1976) and the guidelines prescribed by the National Institutes of Health (1985). New York University Medical Center animal facilities have been approved by the New York University Institutional Animal Care and Use Committee and are registered with the U.S. Department of Agriculture.

TREATMENT REGIMENS

Carbon Monoxide Dose Response

The cockerels were exposed to CO or filtered air for two hours/day, five days/week, from 6 to 22 weeks of age. Exposures were performed in 1.3-m^3 steel and Plexiglas dynamic exposure chambers. The flow rate of inlet air was kept constant at 300 L/min. Static pressure was kept constant in each chamber at 0.7" of water. The CO (minimum purity greater than 99.0%) (AGL, Newburgh, NY) concentration in each chamber was controlled by a compressed gas regulator (model SG3500, Union Carbide, New York, NY) fitted with a dual pattern micrometer valve (Nupro, Willoughly, OH). The CO concentrations were measured four times during each exposure period with an Ecolyzer portable CO monitor (model 2600, Energetics Science, New York, NY) that was calibrated weekly with an ECO span gas monitor. There were four experimental groups (50, 100, 150, and 200 ppm CO) plus two control groups. One of these control groups was exposed in the chambers to filtered air, while the second group (a stress of handling control group) was kept in the animal quarters throughout the experiment and was never moved to the exposure facility.

Every four weeks, blood samples (1 mL) for COHb and cholesterol determinations were drawn into heparizined syringes from one of the alar veins of each bird. For most experiments, there was approximately a forty-five-minute interval between the end of the CO exposures and drawing the blood. The blood samples were taken after the cockerels had been removed from the exposure chambers and returned to the animal quarters. After exposures ended, but before the chambers were opened, at least 20 additional minutes elapsed, during which CO concentrations in the closed chambers returned to ambient levels. The blood

samples were stored in the dark for subsequent CO and hemoglobin analysis.

Carbon Monoxide as a Coatherogen

Animals receiving the standard chick diet supplemented with 0.1% cholesterol were exposed in the chambers to filtered air or 100 ppm CO for two hours/day, five days/week, from 6 to 22 weeks of age. There were three control groups: a stress of handling group (see above), a group exposed daily to filtered air, and one exposed daily to 100 ppm CO and no cholesterol supplement.

Carbon Monoxide and Plaque Augmentation

Cockerels either received weekly intramuscular injections of the polynuclear aromatic hydrocarbon carcinogen, DMBA from 6 to 22 weeks of age or were fed their standard diet, suppplemented with 1.0% cholesterol, during that age period. All cockerels then were fed a normal diet. Those that had been injected with DMBA were exposed to 100 ppm CO or filtered air from 23 to 34 weeks of age. Cholesterol-fed animals were exposed to 100 ppm CO or filtered air from 23 to 44 weeks of age. The DMBA was dissolved in dimethylsulfoxide (DMSO). The injected dose was 10 mg/kg body weight. The weekly injections were made alternately into the left and right pectoral muscle. Groups injected with DMSO were included as controls for the cockerels injected with DMBA. Three groups (one each from cholesterol, DMBA, or DMSO treatment) were killed at 22 weeks of age to provide baseline values of plaque volume means and ranges. Cockerels exposed to combinations of DMBA and CO or DMBA and air were killed at 34 weeks of age. Cockerels exposed to cholesterol and CO, cholesterol and air or DMSO alone, were killed at 44 weeks of age.

PLAQUE MORPHOMETRY

Plaque morphometry was carried out on abdominal aorta plaques from all the cockerels in each test and control group (6 to 8 per group for the dose-response studies and 6 to 10 per group in the coatherogen and plaque augmentation studies). Immediately after the cockerels were killed (by cervical dislocation under light anesthesia), each aorta was removed, washed in warm buffered saline, cleaned of excess connective tissue, and fixed in phosphate-buffered formalin (pH 7.4). Each aorta was coded before sectioning, and the code was not broken until all relative plaque volumes had been determined. Aortas were processed by the method described previously (Penn et al. 1980). Briefly, the entire aorta from each cockerel was sectioned transversely into 5-mm segments from the iliac arteries to the thoracic aorta. Following paraffin embedding, 5-μm-thick sections were cut from each 5-mm segment and stained by the Verhoeff-van Gieson procedure (Humason 1972), which identifies elastin and collagen and allows the medial (smooth muscle cell) layer and the outer adventitial (connective tissue) layer of the artery wall to be distinguished readily from each other and from plaque, where it exists.

At the suggestion of one of the members of the Health Effects Institute Research Committee, we altered our original procedure for determining plaque size (Penn et al. 1980, 1981a,b) in order to eliminate processing-based biases (see below). With the help of Dr. Thomas Zeltner, then of the Harvard School of Public Health, we developed a modified point counting procedure (Weibel 1979) that overcomes these problems. By determining the plaque cross-sectional area relative to that of the underlying medial region for each consecutive segment of the aorta, we could measure the relative volume of the arterial wall that is occupied by plaque.

A magnified (× 250) image of the arterial section was projected onto a viewing screen attached to a Zeiss microscope. A grid of equally spaced points, 10 mm apart, was superimposed upon the magnified image. The points falling on the plaque (P_1) and the points falling on the underlying medial (smooth muscle cell) region of the artery wall (P_2) were counted. For any section cut from an artery, $P_1/(P_1 + P_2)$ provided an unbiased estimate of the fraction of the arterial wall cross sectional area occupied by plaque. Artifactual problems resulting from fixation and sectioning were minimized because the entire section (wall and plaque) was treated as a single unit. Were only cross sectional plaque area to be measured, subtle changes in the angle of sectioning from segment to segment could have major effects on the calculated cross sectional area. Also, it is sometimes difficult to determine exactly where plaques begin and end. Both of these problems are overcome by this point counting method.

Operationally, each section was scanned in a stepwise manner. The grid, with points 10 mm apart, was made on a fixed, unexposed sheet of autoradiography film and taped to the viewing screen. Because the medial region of the aorta is generally much thicker than any plaque, the media was scanned so that for every 16 points on the grid that appear in the field of view, only one was counted. The sum of the media points then was multiplied by 16. For plaques, all grid points that appeared when the grid was superimposed upon the plaque were counted. The entire section was scanned, regardless of whether it was circular or cut and flattened (Figure 1). The plaque volume percentage is the percentage of the aortic wall (plaque and media) that is occupied by plaque. For each animal, a minimum of 480 points were counted on at least nine sections. This is well in excess of the minimum numbers suggested for accurate analysis (Gundersen 1986).

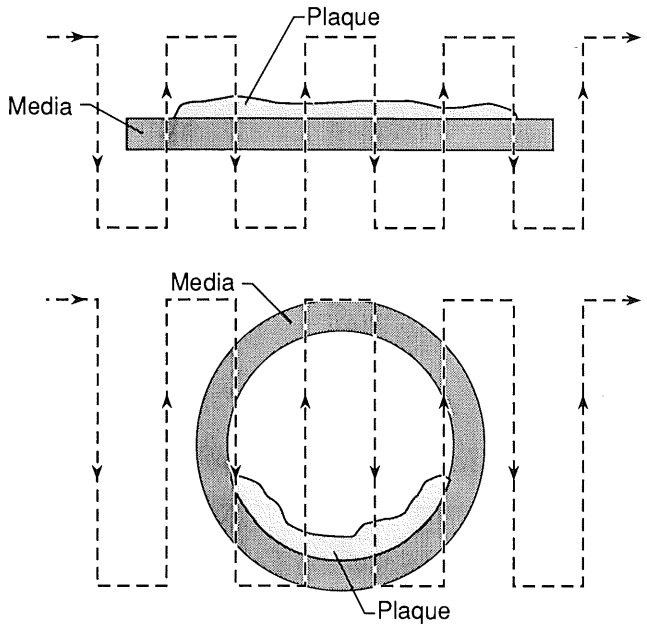

Figure 1. Diagram representing the point-counting grid overlying an aortic section in which an arteriosclerotic plaque is represented.

METHODOLOGY FOR ANALYSIS OF CARBON MONOXIDE IN WHOLE BLOOD SAMPLES

Originally, we had proposed to employ a spectrophotometric method to analyze blood COHb. However, because the concurrent HEI-sponsored clinical CO studies employed gas chromatography (GC) analysis of CO, we agreed to the request from HEI to try to employ the same GC methodology. We adapted and modified extensively the vortex method for blood gas extraction and GC analysis described originally by Hackney (1967) and in greater detail by Dahms and Horvath (1974) (see Appendix A). Unfortunately, serious technical problems arose with the GC method (see the Results section), and this approach was abandoned.

When the GC method proved to be unsuitable for our purposes, we selected a two-wavelength spectrophotometric method (Dijkuizen et al. 1977) to measure COHb in the blood of cockerels that had been exposed to CO. In a previous set of studies, we had exposed cockerels to either cigarette smoke or 50 ppm CO (Penn et al. 1983). In that study, we monitored chamber CO levels and used this spectrophotometric procedure to measure blood COHb levels.

In this procedure, the blood sample is treated as two components (COHb/oxyhemoglobin) and is analyzed spectrophotometrically at two different wavelengths, 562 nm and 540 nm. The latter is isobestic for COHb and oxyhemoglobin. The validity of this procedure over a very wide range of COHb values (0% to 90%) was confirmed by converting CO (from COHb solutions of different concentrations) to CO_2 and titrating this at pH 10 with a sodium hydroxide solution of known strength. The samples were analyzed spectrophotometrically at the two wavelengths. A least squares linear regression line was constructed of absorbance ratio (at the two wavelengths) versus percentage of COHb. From this, an equation for determining the COHb fraction was derived: COHb fraction = $3.215 \times (A_{562}/A_{540}) - 1.923$.

CHOLESTEROL DETERMINATIONS

Cholesterol measurements were made four times on serum samples of cockerels fed a standard diet supplemented with 0.1% cholesterol from 6 to 22 weeks of age. Samples were taken at 6 (baseline), 9, 22, and 44 weeks of age. Cholesterol levels were measured with a Sigma kit (Sigma, St. Louis, MO).

The principles and procedures for this determination are fully discussed by Bergmeyer (1974). In this procedure, cholesterol esterase catalyzes the conversion of cholesterol esters into free cholesterol, which, in the presence of oxygen and cholesterol oxidase, is converted into 4-cholestenone and hydrogen peroxide. The hydrogen peroxide plus 4-aminoantipyrene and *p*-hydroxybenzene sulfonate, in the presence of peroxidase, is converted to a quinoneimine dye and water. The dye is detected spectrophotometrically at 500 nm.

DATA ANALYSIS

Statistical analyses were applied to three groups of variables: plaque volume percentages, serum cholesterol levels, and percentage of COHb levels, all of which are reported as means ± SEM. Samples destined for plaque volume analysis were treated as follows: After the humane killing of animals and the removal of aortas, labeled vials containing fixed, excised aortas were provided to a histologist who coded each vial without knowledge of its source. After samples were processed, they all were read in a blinded manner. The code was not broken until plaque volumes for all samples had been recorded. Serum cholesterol and COHb levels also were read in a blinded manner. The slides for Table 1 were read by a different person than were the slides for Tables 2 and 3.

Comparisons of plaque volume percentages (Tables 1, 2, and 3) were made by a one-factor analysis of variance (ANOVA), followed (in Table 3) by Dunnett's *t*-test. Paired comparisons of rat COHb levels (Table 7) were made by the Student's *t*-test. Comparisons of serum cholesterol levels (Table 4) and of cockerel COHb levels at different times (Table 8) were made by ANOVA for repeated measures, followed by the Student's Newman-Keuls test. The sig-

nificance level was $p < 0.05$. The Super ANOVA (Macintosh) program (Apple Computer Corp., Cupertino, CA) was used for the repeated measures ANOVAs.

RESULTS

CHAMBER CARBON MONOXIDE MEASUREMENTS

The CO concentrations in each chamber were monitored four times per day during each exposure period. For the dose-response study below, the mean ± SEM values for the 77-day exposure periods were: for 50 ppm CO, 50.7 ± 0.5; for 100 ppm CO, 100.5 ± 0.5; for 150 ppm CO, 148.2 ± 1.7; and for 200 ppm CO, 202.1 ± 0.9.

For the second set of studies (CO as a coatherogen), with 78-day exposure periods), the average measured 100 ppm CO value was 99.4 ± 0.7.

For the third set of studies (CO in plaque augmentation, with 97-day exposure periods), the average measured 100 ppm CO values were 100.7 ± 0.9 and 100.8 ± 0.6 ppm (2 chambers were used).

CARBON MONOXIDE DOSE RESPONSE

Six groups of cockerels were tested. Carbon monoxide exposures were made at concentrations of 50, 100, 150, and 200 ppm, two hours/day for 16 weeks, beginning at six weeks of age. There were two control groups. One group (air controls) received filtered air in an exposure chamber at the same time that all the test cockerels were being exposed to CO. Cockerels in the second group (cage controls) remained in the animal facility while all other cockerels were being exposed. This latter group served as controls for possible stresses that might have arisen in the other groups during daily handling and exposures. At the start of the experiment, there were seven cockerels for each group, except the cage controls, which had eight. During the experiments, one cockerel died from each of the following groups: air, 50 ppm, and 150 ppm. They were not included in the analyses. For each cockerel, 10 contiguous aortic segments were cut from the iliac bifurcation, rostrally to the start of the thoracic aorta. The relative plaque volumes were calculated for each group. The dose-response results are summarized in Table 1.

Regardless of the CO level tested, there was no discernible effect of CO on aortic plaque development. Mean aortic plaque volume percentages ranged from 2% to 4%. There were no significant differences between groups (one-factor ANOVA, $p > 0.05$). The slightly elevated values for means and variances in the air control and 100 ppm groups were due to the presence of one outlier in each of these two groups. When these values were omitted, the means dropped to 2.49 and 2.17, respectively. However, because the number of animals in each group was low and there were no significant differences among groups even when the higher values were included, all the values were retained. These results clearly demonstrate that in this animal model, CO administered two hours/day for 16 weeks, at levels as high as 200 ppm, has no detectable effect upon the development of arteriosclerotic plaques. These results reinforce our previous observation that CO alone at 50 ppm has no effect upon plaque development in cockerels (Penn et al. 1983).

Table 1. Mean Aortic Plaque Volume Percentages of Cockerels in the Carbon Monoxide Dose-Response Study

Carbon Monoxide Dose[a]	n	Plaque Volume Percentage[b]
Cage controls[c]	8	2.70 ± 0.43
Air controls	6	4.05 ± 1.63
50 ppm	6	1.97 ± 0.30
100 ppm	7	4.36 ± 2.27
150 ppm	6	2.78 ± 0.50
200 ppm	7	2.49 ± 0.63

[a] The duration of exposures was two hours/day, five days/week, for 16 weeks.

[b] Plaque volume percentage is the percentage of aortic wall (plaque and media) occupied by plaque. Each value is expressed as a mean ± SEM. There were no significant differences among plaque volume means ($p > 0.05$, one-factor ANOVA).

[c] Cage-control animals were kept in the animal facility at all times and never were exposed in the chambers. They represented a control for the stresses of daily handling and transfer.

CARBON MONOXIDE AS A COATHEROGEN

In the second stage of the program we investigated whether CO treatments administered at the same time that the diet was supplemented with a low level of cholesterol resulted in plaque sizes that were larger than those seen following exposure to either agent alone. From 6 to 22 weeks of age, cockerels were fed a standard diet supplemented with 0.1% cholesterol and were exposed daily (two hours/day) in the inhalation chambers to either filtered air or to 100 ppm CO. (Because the dose-response results demonstrated that 100 and 200 ppm CO alone were equally ineffective at promoting plaque development, and because 100 ppm CO is at the high end of the range of measured urban CO values, we selected 100 ppm CO as the exposure level for this second set of experiments). As shown in Table 2, CO administration resulted in no increase in plaque size in cockerels fed the cholesterol-supplemented diet. Mean aortic plaque volume percentages ranged from 5% to 7%. There were no significant increases in plaque size ($p > 0.05$) in the CO plus cholesterol group in comparison with the CO, air control, or

Table 2. Mean Aortic Plaque Volume Percentages of Cockerels Exposed to 100 ppm Carbon Monoxide, With and Without Concomitant Feeding of Low Levels (0.1%) of Cholesterol

Exposure Group[a]	*n*	Plaque Volume Percentage[b]
Cage controls	10	4.99 ± 1.34
Air controls	8	6.53 ± 1.54
CO (100 ppm)	9	5.62 ± 1.45
Cholesterol (0.1%)	9	7.32 ± 1.92
CO (100 ppm) plus cholesterol (0.1%)	9	6.45 ± 1.25

[a] The duration of exposures was two hours/day, five days/week, for 16 weeks (6 to 22 weeks of age).

[b] Plaque volume percentage is the percentage of aortic wall (plaque and media) occupied by plaque. Each value is expressed as a mean ± SEM. There were no significant differences among plaque volume means ($p > 0.05$, one-factor ANOVA).

cholesterol groups alone, although cockerels exposed only to CO had less aortic plaque involvement than cockerels in any of the other three groups. The means of the cage control, air control, and 100 ppm (no cholesterol) groups in Table 2 are all higher, although still within the limits of experimental error, than the comparable values from Table 1. The plaque sizes in these first two sets of studies were very small. Because a different person made each of these sets of measurements, variations between the comparable values in the two tables probably resulted from systematic differences in measurements from viewer to viewer.

ROLE OF CARBON MONOXIDE IN PLAQUE AUGMENTATION

In the third stage of this program, we asked whether CO would further augment plaque development if administered chronically to animals in whom plaque development had been stimulated previously.

Animals were fed a diet containing 1% cholesterol from 6 to 22 weeks of age, or were injected weekly with 10 mg/kg of DMBA during the same time period. After cholesterol feeding and DMBA injections were completed, one-half of each group was exposed in the chambers to 100 ppm CO daily for two hours, and the other half of each group was exposed in the chambers to filtered air for two hours. A separate set of control animals, injected with DMSO, the solvent for the injected DMBA, was also included in the study.

In Table 3, the treatment type and duration, plaque volume percentages, and ranges of plaque volume are presented. Differences among the means of each group were compared via a one-way ANOVA using Dunnett's *t*-test. Although there are apparent differences in relative plaque volumes among many groups, these differences were often not significant at the level tested ($p>0.05$), due to the large range of values. For example, there was no significant difference between the mean values for groups 1 and 2.

Originally, we had planned to expose all animals in the second half of each experiment (i.e., to CO or air) until they were 44 weeks old. However, some of the DMBA-treated animals became seriously and unexpectedly ill during the 33rd week. In all previous experiments, cockerels injected with 10 mg/kg of DMBA from the ages of 6 to 22 weeks re-

Table 3. Effect of Chronic Carbon Monoxide Inhalation on Augmentation of Plaque Development in Cockerels Previously Fed Cholesterol-Supplemented Diets or Injected with DMBA: Comparison of Mean Values and Ranges of Plaque Volume Percentages

Group	Treatment	Plaque Volume Percentage	
		Mean ± SEM	Range[a]
1	1% Cholesterol, killed at 22 weeks (n = 7)[b]	8.49 ± 2.74[c]	1.66–19.20 (4.92)
2	1% Cholesterol, CO, killed at 44 weeks (n = 8)[d]	17.62 ± 4.80	5.71–46.45 (12.08)
3	1% Cholesterol, Air, killed at 44 weeks (n = 7)[d]	19.63 ± 4.67	2.30–31.95 (21.74)
4	DMBA, killed at 22 weeks (n = 6)[b]	10.44 ± 2.20[c]	5.65–15.95 (9.90)
5	DMBA, CO, killed at 34 weeks (n = 6)[e]	24.42 ± 4.79	3.15–37.05 (25.33)
6	DMBA, Air, killed at 34 weeks (n = 4)[e]	32.44 ± 7.07[c]	15.56–45.72 (34.24)
7	DMSO, killed at 22 weeks (n = 8)[b]	10.24 ± 3.27[c]	1.12–27.13 (8.84)
8	DMSO, killed at 44 weeks (n = 8)[d]	9.21 ± 1.53[c]	3.69–17.82 (9.14)

[a] Median values are given in parentheses.

[b] Exposures to cholesterol, DMBA, or DMSO alone were from 6 to 22 weeks of age.

[c] Significant differences among groups (one-factor ANOVA, Dunnett's *t*-test, $p < 0.05$) were found only between groups 1 and 6, 4 and 6, 7 and 6, and 8 and 6. A two-factor (experiment, exposure) ANOVA of the CO and non-CO pairs yielded no significant differences between the paired exposure groups.

[d] Cholesterol-fed or DMSO-treated cockerels were exposed to CO or to filtered air from 23 to 44 weeks of age.

[e] DMBA-treated cockerels were exposed to CO or to filtered air from 23 to 34 weeks of age.

mained healthy for the next 20 weeks. The results presented here only include those DMBA-treated cockerels that were healthy when killed at 34 weeks of age. Despite this shortcoming, we obtained striking results, which are summarized below.

We previously had reported that spontaneous aortic plaques in the cockerel are not significantly larger at 38 weeks of age than they are at 20 weeks of age (Penn et al. 1980). Those results are confirmed and extended here. Plaque volume percentages in control animals were relatively low and nearly identical at 22 weeks (group 7) and 44 weeks of age (group 8). The results were strikingly different in the DMBA-treated groups. Twelve weeks after the end of DMBA injections (i.e., at 34 weeks of age), cockerels in the DMBA-air group (group 6) had relative plaque volumes that were significantly larger (three times greater) than those displayed by the 22-week-old cockerels in group 4 (i.e., immediately after DMBA injections had ended). Although this delayed increase in plaque size was not unexpected, the lack of a striking DMBA effect immediately after injections ended (group 4) was puzzling. This was especially so because we and others had previously demonstrated that shortly after multiple carcinogen treatments end, plaque sizes in young birds are significantly greater than in controls (Albert et al. 1977; Penn et al. 1981a,b; Batastini and Penn 1984; Revis et al. 1984; Majesky et al. 1985; Penn and Snyder 1988).

Cockerels pretreated with DMBA or 1% cholesterol and then exposed to CO starting at 23 weeks of age (groups 5 and 2, respectively) were compared with cockerels pretreated similarly and then exposed to filtered air (groups 6 and 3, respectively); there were no significant differences between the respective means (group 6 versus group 5, and group 3 versus group 2). In both cases, the mean plaque volumes for CO-exposed cockerels were actually lower than the values for control cockerels exposed to filtered air. However, because of the wide variance within each group, the differences were not significant ($p>0.05$). Thus, 20 weeks of exposure to CO after 16 weeks of cholesterol feeding yielded plaques no larger than those obtained with 20 weeks of air exposure following 16 weeks of cholesterol feeding. Similarly, 11 weeks of CO exposure following 16 weekly DMBA injections was, if anything, less effective than 11 weeks of air exposure at augmenting DMBA-stimulated plaque development. In summary, in this animal model, further development of preexisting plaques is not augmented by daily (two hours/day) exposure to 100 ppm CO.

In addition to testing the effect of CO inhalation on plaque development, we also tested its effects on serum cholesterol levels. The latter were determined four times during the third set of experiments. Two groups of cockerels were sampled: those fed a 1% cholesterol-supplemented diet for 16 weeks and then (1) exposed to filtered air and killed at 44 weeks of age; or (2) exposed to CO and killed at 44 weeks of age. Serum cholesterol levels were measured at zero time, three weeks into the 16-week feeding period, and one week and 22 weeks after the end of the cholesterol feeding period. Results are summarized in Table 4. Data were analyzed by a repeated measures ANOVA followed by the Student's Newman-Keuls test. Within three weeks, there was a statistically significant twofold increase in serum cholesterol levels in both sets of animals. One week after cholesterol-supplemented feeding ended, there was a statistically significant 23% to 28% decrease in serum cholesterol levels in both groups ($p<0.05$). After 22 weeks of CO or filtered air exposures, there was a further sharp decrease in serum cholesterol levels in both groups. In the air controls, these final values were 25% below baseline, and in the CO-exposed

Table 4. Serum Cholesterol Levels of Cockerels Fed a Diet Supplemented with 1% Cholesterol Followed by Exposure to Carbon Monoxide or Filtered Air

	Age (weeks)[a]			
Treatment Group	6	9	23	44
1% Cholesterol (6 to 22 weeks), CO (23 to 44 weeks)	118 ± 5	248 ± 34	191 ± 25	79 ± 7
1% Cholesterol (6 to 22 weeks), Air (23 to 44 weeks)	115 ± 5	244 ± 28	170 ± 17	86 ± 13

[a] Age 6 weeks = zero time. Age 9 weeks = after three weeks of feeding 1% cholesterol-supplemented diet. Age 23 weeks = one week after ending 16 weeks of 1% cholesterol supplementation. Age 44 weeks = 22 weeks after ending 16 weeks of 1% cholesterol supplementation. Data were analyzed by a repeated measures ANOVA followed by the Student's Newman-Keuls test. Each value is expressed as a mean ± SEM. Significant differences ($p < 0.05$) existed between the group at age six weeks and each of the other three groups in the same treatment regimen, as well as between the group at age 44 weeks and the groups at ages 9 and 23 weeks of the same treatment regimen.

group, the values were 33% lower than baseline values. There were no statistically significant differences among groups at any time point ($p>0.05$). Thus, in this animal model, CO inhalation has no effect either on plaque development or serum cholesterol levels.

CARBOXYHEMOGLOBIN LEVELS

The modified GC system (see Appendix A, Figure A.1) was adequate for single measurements of CO levels. A unique CO peak could be identified in a chromatogram of a mixture of gases, and the CO concentration in the mixture could be quantified. In addition, a very small CO peak could be identified in samples from cockerels breathing 200 ppm CO. This peak was barely present in the blood samples of unexposed animals (Figures A.2 and A.3). However, this system proved to be impractical for large numbers of COHb measurements. Scrubbing columns for this system were no longer available commercially. Their absence resulted in clogging and contamination of the sample column, which had to be stripped and recalibrated after only a few readings. No more than two samples plus standards could be run before the column had to be stripped and the process started again. Despite these technical problems, one series of COHb measurements was completed using this system. Blood samples were analyzed from cockerels exposed to 50 and 100 ppm CO. The results, presented in Table 5, show that none of the measured COHb levels even approached a value of 1%. These values were extremely low considering that the animals had been breathing moderately high levels of CO for the two hours before blood was sampled. To determine whether these low values were due to an analytical error, we sent a separate set of six blood samples from CO-exposed cockerels to two other investigators (Drs. John O'Neil of the U.S. Environmental Protection Agency and Michael Kleinman of the University of California at Irvine) and requested that they measure COHb levels in these samples via gas chromatography. Both sets of blood samples were collected, stored, and shipped according to their requests. Of the six samples, three were from cockerels exposed to 200 ppm CO, and three were from cockerels exposed to filtered air. The two sets of results are presented in Table 6. The results of these two separate COHb determinations mirror our results. The conclusion that emerges from these three sets of analyses is that there was very little COHb in any of the samples that were analyzed.

There are four areas in which problems could arise, resulting in low measured COHb levels: processing, exposure, sampling, and analysis. Processing was eliminated because three different investigators, each using distinct extraction and analytical procedures, obtained results that were virtually indistinguishable. Exposure was eliminated as a possi-

Table 5. Blood Carboxyhemoglobin Levels (as Measured by Gas Chromatography) in Blood Samples from Cockerels Taken After a Two-Hour Exposure to Carbon Monoxide

Sample Number	Carbon Monoxide (μL/100 μL blood sample)	Blood Hemoglobin (g/dL)	Oxygen Carrying Capacity	% Carboxyhemoglobin
50 ppm				
440 and 441	0.023	12.35	17.154	0.13
50 ppm				
437	0.018	12.30	17.085	0.11
439	0.025	12.50	17.363	0.14
440	0.010	12.70	17.640	0.06
441	0.012	12.00	17.668	0.07
442	0.127	8.30	11.529	0.23
100 ppm				
443	0.027	14.00	19.446	0.14
444	0.004	12.00	16.668	0.02
446	0.024	12.30	17.085	0.14
448	0.019	11.90	16.529	0.12
449	0.011	10.00	13.890	0.09
210	0.010	11.80	16.390	0.06
211	0.019	12.60	17.501	0.11
209	0.025	12.60	17.501	0.14
212	0.010	10.60	14.723	0.07
213	0.008	12.00	16.668	0.05

Table 6. Blood Carboxyhemoglobin Levels (as Measured by Gas Chromatography) in Blood Samples from Cockerels Taken After a Two-Hour Exposure to Either 200 ppm Carbon Monoxide or Filtered Air

Sample Number	Set 1[a] % Carboxy-hemoglobin	Set 2[b] % Carboxy-hemoglobin
Carbon Monoxide		
290	0.09	0.14
292	0.27	0.03
293	0.28	$\leqslant$ 0.05
Air		
350	0.15	$\leqslant$ 0.05
352	0.11	$\leqslant$ 0.05
353	0.21	$\leqslant$ 0.05

[a] Samples were analyzed by Dr. John O'Neil of the U.S. Environmental Protection Agency.

[b] Samples were analyzed by Dr. Michael Kleinman of the University of California at Irvine.

Table 7. Blood Carboxyhemoglobin Levels (as Measured by Spectrophotometry) in Blood Samples from Rats Before and After Exposure to 200 ppm Carbon Monoxide

	% Carboxyhemoglobin Levels[a]	
Rat Number	Before Exposure	45 to 60 Minutes After CO Exposure Ended
1	2.20 ± 0.25	10.30 ± 0.21
2	3.50 ± 0.46	9.50 ± 0.12
3	3.90 ± 0.26	11.10 ± 0.39
4	4.20 ± 0.46	9.50 ± 0.52
5	1.70 ± 0.16	5.80 ± 0.08
Mean	3.20 ± 0.45	9.24 ± 0.91

[a] Each value is expressed as the mean ± SEM of five determinations. There were significant differences between mean COHb levels ($p < 0.05$, Student's t-test).

ble source of error because the protocols followed in these studies were very similar to ones we had followed in other CO studies (Penn et al. 1983; unpublished observations). Additionally, CO levels were measured four times during each exposure and, as noted above, the measured chamber CO levels were both precise and accurate. Analysis was essentially ruled out because gas chromatographic analysis of COHb is widely used and highly regarded. However, we could find no reports of gas chromatographic analysis of avian COHb. For this reason, and because previously we had successfully employed a two-wavelength spectrophotometric method (Dijkuizen et al. 1977) to analyze COHb levels, we tested whether this method might yield clues to explain the low COHb levels measured in the blood of CO-exposed cockerels. As a test of the system, a pilot study was carried out on blood from CO-exposed rats. Five adult male Sprague-Dawley rats were exposed to 200 ppm CO for two hours. Blood samples were taken before and after exposure, and five replicate COHb measurements were made spectrophotometrically on each sample. The results are presented in Table 7.

Up to 60 minutes after exposure to CO was completed, COHb levels were 125% to 180% higher than preexposure values in five out of five rats. However, when the same experiment was repeated with cockerels (i.e., blood samples were taken 45 to 60 minutes after the end of exposures), blood COHb levels were all less than 1% (data not presented). Therefore, it appeared that a sampling problem existed. Because chamber levels of CO were maintained at the correct levels throughout the exposures of both rats and cockerels, we investigated whether the anomalously low levels of COHb in cockerels resulted from their clearing CO far more rapidly than expected.

In order to ensure the safety of the animal handlers and to permit blood samples to be taken shortly after the end of exposures (see the Discussion section), the individuals handling the cockerels in the exposure chambers were fitted with respirators. Two groups of cockerels were exposed to 100 or 200 ppm CO. Blood samples were taken within 10 minutes after the end of exposures (100 and 200 ppm), as well as 30 minutes (100 ppm), and 60 minutes (200 ppm) after the end of exposures. Carboxyhemoglobin levels in these samples, determined spectrophotometrically, were compared with preexposure COHb levels. The results of five replicate measurements of each sample are presented in Table 8. Data were analyzed by a repeated measures ANOVA followed by the Student's Newman-Keuls test.

When samples were taken from the 100 ppm group 10 minutes after the end of exposures, there were statistically significant increases in COHb levels. These values were significantly higher (65% to 115%) than their preexposure values ($p<0.05$). However, when samples were taken from the same birds only 30 minutes after the end of exposures, all the COHb levels dropped sharply. There was no significant difference between the preexposure values and the 30-minute postexposure values ($p>0.05$).

When samples were taken in the 200 ppm group within 10 minutes after the end of exposures, there were statistically significant increases in COHb levels ($p<0.05$). The values ranged from 90% to 270% higher than the preexposure values. However, when sampling was carried out 60 minutes after the end of exposures, the measured COHb lev-

Table 8. Blood Carboxyhemoglobin Levels (as Measured by Spectrophotometry) in Blood Samples from Cockerels Before and After Exposure to Carbon Monoxide[a]

Cockerel Number	Before Exposure	10 Minutes After CO Exposure Ends[b]	30 or 60 Minutes After CO Exposure Ends[c]	Calculated Clearance Halftime (minutes)
100 ppm CO				
1	2.96 ± 0.63	9.61 ± 0.35	3.84 ± 0.35	6.8
2	4.02 ± 0.64	5.25 ± 0.63	4.19 ± 0.67	7.0
3	3.62 ± 0.63	5.98 ± 0.13	4.35 ± 0.20	11.7
4	3.82 ± 0.04	6.37 ± 0.25	4.53 ± 0.33	10.8
5	2.92 ± 0.28	6.60 ± 0.30	4.52 ± 0.22	16.5
Mean	3.47 ± 0.22	6.76 ± 0.75	4.29 ± 0.13	
200 ppm CO				
6	3.30 ± 0.63	11.16 ± 0.28	4.36 ± 0.43	17.3
7	4.90 ± 0.55	9.39 ± 0.63	3.52 ± 0.37	16.5
8	4.10 ± 1.15	11.25 ± 0.24	5.44 ± 0.55	13.9
9	4.00 ± 0.37	13.21 ± 0.22	4.69 ± 0.28	13.3
10	3.30 ± 0.37	11.95 ± 0.38	3.19 ± 0.37	8.0
Mean	3.92 ± 0.30	11.39 ± 0.62	4.24 ± 0.41	

[a] Data were analyzed by a repeated measures ANOVA followed by the Student's Newman-Keuls test. Each value is expressed as the mean ± SEM of five determinations.

[b] Significantly different ($p < 0.05$) from preexposure and 30-minute postexposure values for cockerels exposed to 100 ppm CO. Significantly different ($p < 0.05$) from preexposure and 60-minute postexposure values for cockerels exposed to 200 ppm CO.

[c] For 100 ppm CO exposure, COHb was measured 30 minutes after exposure ended; for 200 ppm CO exposure, COHb was measured 60 minutes after exposure ended.

els were not significantly different from the preexposure COHb values in the same birds. Thus, cockerels exposed to 100 or 200 ppm CO for two hours displayed elevated COHb levels, as was expected. However, this increase was transitory, and within 10 to 30 minutes after the end of peak CO exposures, COHb levels were reduced to baseline levels. Therefore, the low COHb levels found in the blood of CO-exposed cockerels was due to a sampling problem. There was an anomalous and extremely rapid dissociation of CO from COHb, which was complete by the time that blood samples were taken.

DISCUSSION

Although death rates from cardiovascular disease have been decreasing for over 30 years, the disease is still the single greatest cause of death in the Western world. Numerous risk factors for cardiovascular disease have been identified, including elevated serum cholesterol, high blood pressure, and cigarette smoking. In recent years, genetic predisposition and environmental pollutants also have been implicated. A variety of biochemical, electrical, and histopathological conditions and symptoms may be manifested in a given human (or animal) suffering from some form of cardiovascular disease. Thus, it is no surprise that a large number of animal models have been developed to study various aspects of this disease.

Over twenty years ago, a histological and ultrastructural study noted the similarities between cockerel plaques and human fibromuscular coronary artery plaques (Moss and Benditt 1970). Subsequent investigations have demonstrated that environmental agents, including carcinogens (Albert et al. 1977; Penn et al. 1981a,b; Batastini and Penn 1984; Revis et al. 1984; Penn and Snyder 1988), viruses (Fabricant et al. 1978, 1983; Minick et al. 1979; Pyrzak and Shih 1987), and mainstream and sidestream cigarette smoke (Penn et al. 1983; Penn and Snyder 1993) markedly accelerate plaque development in avian species. Because the arteriosclerotic plaque is the most common lesion associated with cardiovascular disease, the availability of a reliable, relatively inexpensive animal model for studying plaque development is very encouraging. The avian models, especially the cockerel, are particularly valuable for two other reasons. First, nondietary as well as dietary factors can readily influence plaque development. Second, the effects of environmental factors on plaque development can be detected at an early age, even after only brief exposures. For example, a single, subtumorigenic injection of DMBA into cockerels at five weeks of age resulted in a transient but sig-

nificant increase in intimal smooth muscle cell proliferation within one week. This was followed two weeks later by a significant increase in plaque size (Penn, unpublished observations).

Many investigators, such as Dwyer and Turino (1989), have maintained that CO exposure can predispose at least certain laboratory animals to atherosclerosis. We reasoned that the procedures we developed for studying the stimulation of plaque development in cockerels would enable us to determine, with a high degree of certainty, whether CO exposures stimulate plaque development in this species.

We measured the extent of plaque development in the abdominal aortas of each of 139 cockerels (including controls) exposed chronically to various levels of CO (0 to 200 ppm). The results clearly show that in this animal model, CO exposures for two hours/day had no effect on plaque development. Despite the exquisite sensitivity of cockerel plaques to modulation by environmental agents, CO administered concurrently with a cholesterol-supplemented diet for 16 weeks had no effect on plaque development. Carbon monoxide exposure by itself, at levels as high as 200 ppm for two hours/day for 16 weeks, also did not affect plaque development. Carbon monoxide administered after large plaques had developed, either as a result of cholesterol feeding or DMBA injections, was incapable of further stimulating plaque development. Although the dissociation of COHb is more rapid in this animal system than in mammalian systems, and steady-state COHb levels likely were maintained only while the animals were in the inhalation chambers, we conclude that in this model, CO inhalation likely does not have a major effect upon arteriosclerotic plaque development.

Although inhaling CO for two hours/day for 16 weeks constitutes a relatively brief exposure, there was precedent from our laboratory for effectively employing this exposure scheme. We had demonstrated previously a small but significant increase of plaque size in cockerels that had been exposed in inhalation chambers to mainstream cigarette smoke for 100 minutes/day for 16 weeks (Penn et al. 1983). Recently, we found that inhalation of sidestream cigarette smoke for six hours/day for 16 weeks caused striking acceleration of aortic arteriosclerotic plaque development in cockerels (Penn and Snyder 1993).

One of the most striking (and unexpected) results was the apparent absence of any COHb in the blood of cockerels that had been exposed to CO levels as high as 200 ppm. The GC system that we devised to measure COHb, although less than ideal for analyzing large numbers of samples, was clearly sufficient for identifying COHb in individual samples (Figure A.3). Our inability to detect COHb levels of even 1% in cockerels exposed to 200 ppm CO for two hours (Table 5) prompted us to send blood samples (three each from air-exposed and CO-exposed [200 ppm] cockerels) to two other investigators for COHb analysis by GC. To ensure that the low COHb levels that we detected were not due to extraction problems, we sent whole blood samples, which subsequently were extracted and analyzed. The findings in those two laboratories confirmed our findings. All three sets of samples had low (less than 1%) or negligible levels of COHb. Measurements of CO taken in the chambers four times during each exposure period had confirmed that the cockerels all had been exposed to the appropriate levels of CO. The absence of detectable COHb in these blood samples strongly suggested that the CO had dissociated from the hemoglobin before the blood samples were taken. Although respiration rates are higher for birds than for most mammals, there was no a priori reason to believe that the half-time for COHb dissociation was as rapid as it appeared to be.

The safety precautions followed at the New York University Institute of Environmental Medicine chamber facility, where the CO exposures took place, mandated that the chambers not be opened after the two-hour exposures until the CO concentrations in them had dropped below 15 ppm. With steady-state chamber CO concentrations of 100 to 200 ppm, more than 20 minutes were needed for chamber CO levels to drop below 15 ppm. In addition, the standard procedure followed with animals exposed in these chambers is to return them to the animal facility before any sampling is done. Thus, 40 to 45 minutes usually elapsed from the time that the CO flow to the chambers ended until blood samples began to be taken. To overcome this delay and to take blood samples as soon as full CO exposures had ended, animal handlers were fitted with respirators, and blood samples were taken at the chambers from cockerels exposed to 100 to 200 ppm CO. The results shown in Table 8 clearly demonstrate that moderately high levels of COHb were detected when blood samples were taken from cockerels within 10 minutes of their removal from the chambers. However, within 30 minutes after being removed from the chambers, cockerels displayed COHb levels that had dropped to near baseline values. Within 60 minutes, the COHb levels from cockerels exposed to 200 ppm CO for two hours were indistinguishable from background levels. For cockerels exposed to 100 ppm CO, the half-time for COHb dissociation was approximately 10 minutes. For animals exposed to 200 ppm CO, the half-time for COHb dissociation was approximately 14 minutes.

These determinations were made only on two sets (two hours/day for five days; 100 and 200 ppm CO) of five cockerels each, well after the three sets of experimental exposures had been completed. Thus, we cannot state with certainty which levels of COHb existed in the 16-week exposure groups receiving 50 to 200 ppm CO. However, for

cockerels inhaling 100 or 200 ppm CO for two hours, the COHb levels during peak CO exposures were probably at least as high as the values that were measured in blood samples taken from these animals within 10 minutes after the CO flow to the chambers ended. Unfortunately, cockerels, unlike mammals, apparently clear CO so rapidly that in this model system the effects of residual COHb upon plaque development cannot be judged. This rapid dissociation of COHb in cockerels strongly suggests that COHb formation also should occur very rapidly. Previous studies have shown that with an airflow of 300 to 350 L/min in these 1.3-m^3 chambers, a steady-state level of an added gas is reached within 18 to 20 minutes (Drew and Laskin 1973). Thus, it is reasonable to expect that the cockerels maintained steady-state COHb levels for at least 100 to 110 minutes.

Although there is little doubt that COHb levels were elevated in blood from CO-exposed cockerels, the absolute magnitude of this elevation is more difficult to assess. These COHb levels were determined via a two-wavelength spectrophotometric method (Dijkuizen et al. 1977). Although there are many reports of spectrophotometric determination of COHb, especially via the CO-oximeter, the reliability of these methods at quantifying low levels of COHb has been questioned (Dennis and Valeri 1980). Dahms and associates (1990) also noted that when optical methods are used to measure low levels of COHb, the values are generally quite a bit higher than those obtained when the same samples are analyzed by GC. A comparison of spectrophotometric and GC analyses of COHb is provided by the HEI Multicenter CO Study Team (1989). The baseline COHb levels recorded spectrophotometrically for both rats (Table 7) and cockerels (Table 8) averaged approximately 3.5%. We had expected values less than 1%. Nevertheless, there was a nearly linear increase in COHb levels at 100 and 200 ppm CO, as measured by the spectrophotometric method. The spectrophotometric method is adequate for these types of experiments in which the relative difference in COHb levels between groups, rather than the rigorous determination of specific COHb levels, is of primary interest.

There were six HEI-sponsored projects in which the effects of in vivo CO exposures on the cardiovascular system were investigated. Altogether, three animal models (dog [used in two experiments], rat, cockerel) and two different groups of human volunteers were examined. The responses to either acute or chronic exposure to CO across a wide range of concentrations (10 to 500 ppm) were recorded. Both healthy subjects and those representative of an increased risk for some manifestation of cardiovascular disease were included. Although a variety of end points were chosen, the investigators reported little or no effect of CO inhalation on any of these end points.

In the animal studies, McGrath (1989) administered moderate to high levels of CO (up to 500 ppm) to male rats in barometric chambers in which altitudes of 3,300 to 18,000 feet were simulated. At CO levels less than 100 ppm, all hemodynamic, hematological, and morphological changes could be attributed to responses to altitude. Significant increases in left ventricular weight occurred only at CO levels greater than 100 ppm.

Using a dog model, Verrier and colleagues (1990) investigated the effect of a single exposure (90 to 120 minutes) to very high levels of CO (500 ppm) on the electrical stability of normal and ischemic hearts. Blood COHb levels rose to approximately 20%. However, no effects were found with regard to ventricular electrical stability or platelet aggregatibility; also, the effect of platelet aggregatibility on coronary blood flow during stenosis was not changed.

Farber and colleagues (1990) investigated the effects of moderately high levels of CO on the induction of lethal arrhythmias in both normal dogs and dogs that had recovered from a previous myocardial infarction. They found that blood COHb levels rose to 5% to 15%. High levels of CO caused increases in heart rate, both in exercise and at rest, but seldom caused arrhythmias, even in dogs with healed myocardial infarctions.

In the human studies, Horvath and coworkers (1988) studied the effects of CO inhalation on maximal aerobic capacity in human volunteers. Healthy nonsmokers (11 males, 12 females) were exposed to CO (0 to 150 ppm) at simulated altitudes of 50 to 3,000 meters. Although COHb levels rose as expected, there was no significant effect of CO exposure on maximal aerobic capacity.

In the very thorough study by the HEI Multicenter CO Study Team (1989; Allred et al. 1989), the effects of CO inhalation on time-to-onset of angina symptoms and on time-to-development of ischemic ST-segment changes were measured in male volunteers, all of whom displayed at least one major clinical symptom of cardiovascular disease. One hour of exposure to CO at a level sufficient to increase blood COHb to either 2% or 4% was followed by an exercise period. The reports show that CO exposures resulted in significant but very limited decreases in the time to onset of ST-segment depression and to the onset of angina symptoms in exercising subjects with preexisting coronary artery disease. There was only an average 4% decrease in the time of onset of angina at 2% COHb. In addition, there was a wide range of absolute values reported by the three centers, all of which followed the same standardized methodology. (At 2% COHb, time-to-angina changes ranged from −3.2% to 9.4%, and time-to-ST-segment changes ranged from −1.1% to 10.8%).

Although CO exposures have proven to be relatively ineffective at exacerbating or accelerating processes and conditions associated with cardiovascular disease, these six HEI-sponsored studies are still very valuable. They point out the importance of studying a variety of animal and patient groups in order to maximize the chances of identifying clinically important responses to exposure to environmental pollutants. In vitro studies, experiments with only one animal model, or computer-generated models would not have been sufficient to test the effects of CO on cardiovascular disease. There was no a priori reason to believe that any one of these six studies would be more effective than the others at demonstrating the cardiovascular disease-promoting effects of CO exposure at environmentally accessible levels. Although the study by the HEI Multicenter CO Study Team (1989) was the only one to report positive findings, the demonstration that low levels of CO can hasten the onset of ischemic episodes justified the multisystem approach for studying environmentally related health conditions.

CONCLUSIONS

In this animal model, chronic exposures to CO (50 to 200 ppm, two hours/day, five days/week, for 10 to 22 weeks) had no detectable effect upon augmentation of arteriosclerotic plaque size or regression of plaques. Although COHb levels were high shortly after exposures ended, the rapid dissociation of COHb in this species resulted in an accelerated return to baseline levels, with little residual COHb effect. Mammals treated to the same exposure regimen would be expected to display higher COHb levels for longer periods of time. Thus, while these results suggest that elevations in COHb levels have little effect on plaque development they do not permit direct extrapolations to mammalian systems.

ACKNOWLEDGMENTS

The results described in this report were derived from the thorough, dependable, and dedicated work of Mr. James Currie. Mr. John Gorczynski is also acknowledged for his imaginative reviving and replumbing of an old gas chromatograph, to convert it into a CO analyzer. Dr. Carroll Snyder, the Director of the Inhalation Chamber Facility at New York University Medical Center Institute of Environmental Medicine provided invaluable assistance with experimental design and data analysis. Drs. Patrick Kinney, John Graefe, and Lung Chi Chen provided excellent help with statistical analyses. I am very appreciative of the interest and assistance provided by the following individuals: Dr. Joe Brain, who first pointed out some problems inherent in the original method of analyzing data, and then provided an introduction to Dr. Thomas Zeltner, who was very generous in describing the theory and practice of point counting; and Drs. John O'Neil and Michael Kleinman, who provided independent confirmation of our results of COHb analysis by gas chromatography.

The secretarial assistance of Ms. Eileen Santan and Ms. Francine Lupino is also acknowledged.

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APPENDIX A. Gas Chromatograph Modified for Carbon Monoxide Analysis

The apparatus we constructed is represented schematically in Figure A.1. The gas chromatograph (h) was a Varian model 1420 equipped with a thermal conductivity detector. Stainless-steel fittings were used throughout. A prepacked molecular sieve 5A stainless-steel column (i) (2 m long, 1/8 inch o.d., 45/60 mesh) was used for the separation of gases. Flow rates through the column for the helium carrier gas (a) were tested by means of a milliflow controller, a magnehelic meter, and a Gasmet flow meter. The Gasmet flow meter was calibrated in turn against a bubble flow meter. Gas-flow rates were set and periodically monitored with the Gasmet flow meter (j).

Carrier gas line pressure, regulated by means of a two-stage pressure regulator (b), was maintained at 60 psi. The helium (ultra-high purity, minimum 99.999%) passed through a Hydro-Purge II filter (c) before entering the gas chromatograph. Carrier gas-flow rate was controlled by means of the milliflow controller (d).

A four-port Valco zero-dead-volume rotary valve (e) was mounted externally and used to control alternately the carrier gas flow either directly to the column or to the column via the vortex blood gas extractor. Tubing of 1/16-inch (o.d.) was used instead of 1/8-inch (o.d.) tubing in order to minimize the amount of dead space. The extraction device consisted of a small (1-mL, 12- × 32-mm) clear-glass vial (Wheaton) (f) sealed with a rubber stopper (West Co., Phoenixville, PA). Rapid stirring of the extraction reagents and sample to facilitate deaeration and gas exchange was accomplished by adding a small stirring magnet to the vial, which was placed on a magnetic stirrer (Tek Pro-Tek Stir 20) (g). A dual stainless-steel injector needle was fabricated by carefully soldering together two 21-gauge needles. This dual injector needle was used to provide an inflow of helium carrier gas to the vial and an outflow of carrier gas and reaction gases to the column. Separate 21-gauge needles (one attached to an inflow line, the other free) were used to purge the reaction vial with helium before introducing the standard gas or blood sample. The standard gas (10% CO in helium, primary standard) and blood samples (generally 100 µL) were injected into the vial using gas-tight high-performance syringes (Hamilton 1000 series).

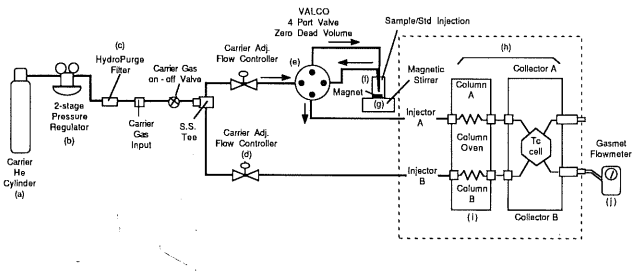

Figure A.1. Schematic diagram of the GC system developed for analysis of CO in cockerel blood samples. The carrier helium was ultra-high purity. Stainless-steel tubing (1/16") was used throughout, and stainless-steel bulkheads (1/8") were used for injectors A and B. Column A was a reference column, and Column B was a prepacked molecular sieve 5A stainless-steel column (2 m, 45 to 60 mesh). Optimal flow through the flow controller was 40 to 70 mL/min (Gasmet) and 45 to 760 mL/min (bubble flow meter). A CO peak was detectable at a column oven temperature of 60°C, but not at ambient temperatures.

The detailed extraction procedure was as follows: Before sealing the vial containing the magnet, the following reagents were added: 100 µL of 2-octanol, 300 µL of lactic acid (85% syrup), and 100 µL of saponin-ferricyanide solution (2 g saponin and 8 g potassium ferricyanide in 42 mL of distilled water). After the vial was sealed, it was purged with ultra-high-purity helium for a minimum of three minutes with rapid stirring. The vial then was attached to the extraction apparatus by piercing the rubber stopper with the dual injector needle. The standard gas or blood sample was introduced using the appropriate gas-tight microsyringe. The sample was extracted for a minimum of three minutes. The gas phase then was eluted onto the column by activating the rotary valve for 60 seconds. Longer activation times did not result in increased CO elution.

The following GC parameters were modified systemati-

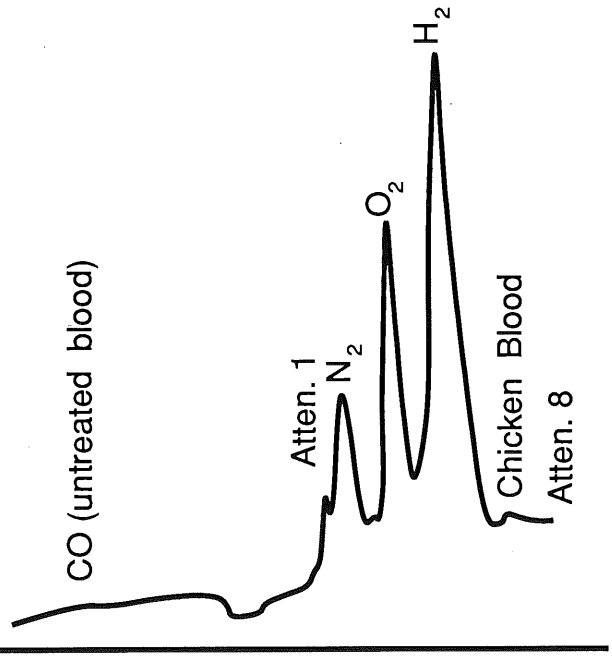

Figure A.2. Gas chromatogram of gases present in 100 µL of freshly drawn blood from a control cockerel breathing filtered air. The peaks for nitrogen, oxygen, and hydrogen were attenuated eight times more than the CO peak (present at the left edge of the trace). Detector current = 200 mA.

cally in an attempt to optimize the conditions for CO elution: temperature, carrier gas-flow rate, detector sensitivity and current, and recorder speed.

Two column temperatures were tested: ambient temperature, which did not yield a CO peak; and 60°C, which was satisfactory. Detector temperature was maintained at 20°C to 25°C above the column temperature.

The helium flow rates tested ranged from 30 mL/min, Gasmet (34.3 mL/min, bubble flow meter) to 92 mL/min, Gasmet (98.5 mL/min, bubble flow meter). Optimal flow was found to lie between 40 mL/min, Gasmet (44.7 mL/min, bubble flow meter) and 70 mL/min, Gasmet (75.8 mL/min, bubble flow meter). The elution time for CO (i.e., length of time between opening of the valve and attainment of a CO peak on the chromatogram) for this range of flow rates was from 5 to 5.5 minutes for flows in the mid-40s (mL/min) to 3.75 to 4.25 minutes for flows in the low- to mid-70s (mL/min).

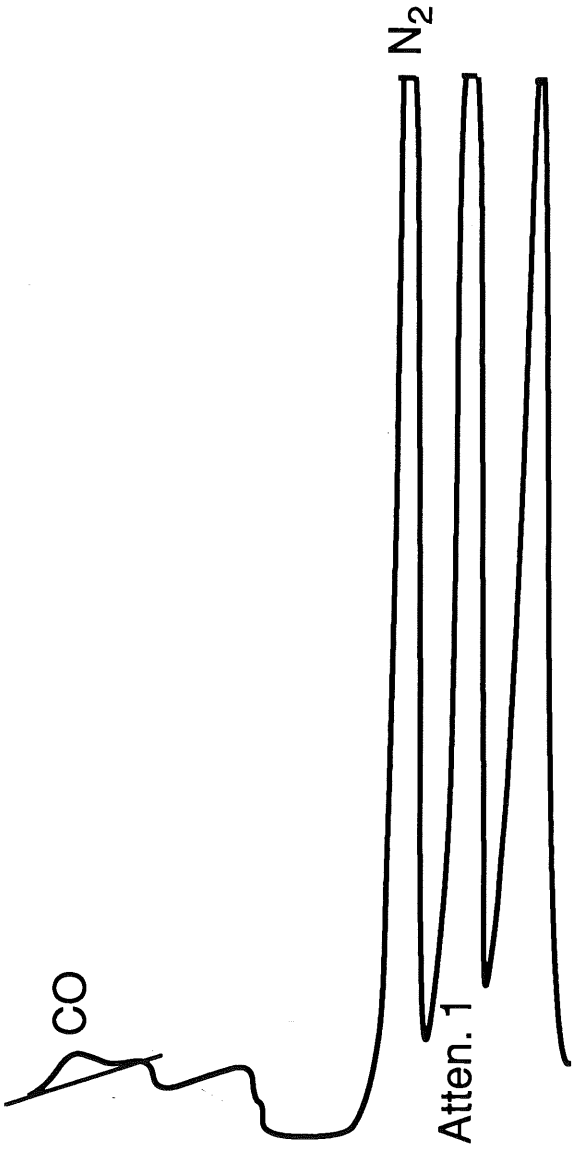

Figure A.3. Gas chromatogram of gases present in 100 µL of blood drawn from a cockerel exposed two hours to 200 ppm CO. The blood sample was taken 45 minutes after the end of CO exposure. The hydrogen peak was attenuated eight times more than the peaks for oxygen, nitrogen, and CO. Detector current = 200 mA.

Three strip-chart recorder speeds were tested: 0.2, 0.5, and 1.0 inches/min. The least flattened and most pronounced blood CO peak heights were attained with a chart speed of 0.5 inches/min.

The detector currents tested ranged from 100 to 250 mA. The optimum detector current for CO measurements was slightly higher than 225 mA.

Two strip-chart recorders were tested. The first was a model A-25 Varian. When set at 1.0 mV full-scale deflection, no CO was detected in any blood samples with this recorder, even with a detector current of 150 mA. The second recorder was a Hewlett-Packard 7101B (Hewlett-Packard Co., Palo Alto, CA) set on 0.1 mV full-scale deflection. This more sensitive recorder enabled us to detect CO in untreated blood samples with a detector current as low as 150 mA. However, at this level, the CO peak appeared as only a barely perceptible rise above the baseline (Figure A.2). A detector current of more than 200 mA was required in order to obtain a more clearly defined CO peak (Figure A.3). Chart paper used for this recorder was 28-cm wide (25.5 cm, gridded).

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PUBLICATIONS RESULTING FROM THIS RESEARCH

Penn A, Currie J, Snyder C. 1991. Inhalation of carbon monoxide does not accelerate arteriosclerosis in cockerels. Eur J Pharmacol 228:155–164.

ABBREVIATIONS

ANOVA	analysis of variance
CO	carbon monoxide
COHb	carboxyhemoglobin
DMBA	7,12-dimethylbenz[*a*]anthracene
DMSO	dimethylsulfoxide
GC	gas chromatography
ppm	parts per million

INTRODUCTION

In September of 1983, the Health Effects Institute (HEI) issued a Request for Applications (RFA 83–1) soliciting proposals for studies of "Cardiovascular and Other Health Effects of Carbon Monoxide." In response to this RFA, Dr. Arthur Penn of New York University Medical Center submitted a proposal entitled "Definitive Determination of the Atherogenic Potential of Inhaled Carbon Monoxide."

The major objective of the RFA was to clarify the effects of carbon monoxide (CO)* exposure on the cardiovascular system, particularly the effects of exposure to CO at ambient levels. The HEI was interested in proposals for research in five categories, including studies of the effects of long-term exposure to high concentrations of CO on atherosclerosis and myocardial function. At the time that RFA 83–1 was issued, there were reports that coronary artery disease was elevated in animals that were fed high cholesterol diets and exposed to CO. There was, however, little information available about the effects of CO on atherosclerosis under environmentally relevant conditions.

Three studies were funded under RFA 83–1. Two were studies of the influence of altitude on CO-induced cardiovascular effects; the third study was that of Dr. Penn, which examined the atherogenic potential of inhaled CO in cockerels (young roosters). Dr. Penn's study began on October 1, 1986, and ended on May 31, 1988. Total expenditures were $298,072. The Investigator's Report was received at HEI in March 1991 and accepted by the Health Review Committee in December 1991.

During the review of the Investigator's Report, the Review Committee and the investigator had the opportunity to exchange comments and to clarify issues in the Investigator's Report and in the Review Committee's Commentary. The following Commentary is intended to serve as an aid to the sponsors of HEI and to the public by highlighting both the strengths and limitations of the study and by placing the Investigator's Report into scientific and regulatory perspective.

REGULATORY BACKGROUND

Section 109 of the Clean Air Act, as amended in 1990, mandates that the U.S. Environmental Protection Agency (EPA) establish primary and secondary National Ambient Air Quality Standards (NAAQS) for air pollution based on the pollutants' health effects at levels "requisite to protect the public health . . . allowing an adequate margin of safety." The Senate Report on the 1970 Clean Air Act Amendments states that "[a]n ambient air quality standard . . . should be the maximum permissible air level of an air pollution agent or class of such agents (related to a period of time) which will protect the health of any group of the population" (U.S. Senate 1970).

Although this reference to "the health of any group of the population" is not clearly defined in the Clean Air Act itself or in the Senate Report, the Senate Report does specify that "included among those persons whose health should be protected by the ambient standard are particularly sensitive citizens (such as bronchial asthmatics and emphysematics) who in the normal course of daily activity are exposed to the ambient environment."

The Senate Report also states that "in establishing an ambient standard necessary to protect the health of these persons, reference should be made to a representative sample of persons comprising the sensitive group rather than to a single person in such a group."

The current primary NAAQS for CO is 9 parts per million (ppm) averaged over eight hours, and 35 ppm averaged over one hour, both not to be exceeded more than once a year (U.S. Environmental Protection Agency 1985). As part of its periodic reevaluation of the criteria pollutant standards mandated by Section 109(d)(1), the EPA reviewed several recent studies of the effects of exposure to CO on the human cardiovascular system. The agency released an external review copy of its draft document, "Carbon Monoxide Air Quality Criteria," on March 29, 1990 to solicit public comment; the draft Staff Paper of the Office of Air Quality Planning and Standards, which recommended no changes in the CO standards, was circulated for technical review and comment in April of 1992 and accepted by the Clear Air Scientific Advisory Committee in August of 1992.

SCIENTIFIC BACKGROUND

The lethal consequences of exposure to high concentrations of CO are well known; however, exposures to low levels of CO are also a public health concern. There is increasing evidence that inhaling small amounts of CO has adverse consequences, particularly for people with heart disease.

CARBON MONOXIDE

Carbon monoxide, an imperceptible poisonous gas, is produced by the incomplete combustion of organic sub-

* A list of abbreviations appears at the end of the Investigator's Report for your reference.

stances such as gasoline, wood, kerosene, natural gas, and other fuels. Exposures may come from indoor and outdoor sources. Outdoor exposures occur largely from motor vehicle exhaust, but fires, industrial processes, and other combustion operations also are sources. In rural areas, background levels of CO are usually less than 1 ppm. Urban CO levels vary from 2 to 30 ppm near roadways (Akland et al. 1985; Bevan et al. 1991; Chan et al. 1991) to levels in excess of 40 ppm in highway tunnels (Stern et al. 1988). Indoor concentrations of CO are usually lower than 2 ppm, but higher levels (10 to 20 ppm) can occur in parking garages and other indoor environments associated with transportation sources (Akland et al. 1985). Indoor CO can reach dangerous levels when appliances or equipment malfunction or when combustion takes place in a poorly ventilated space. Cigarette smoke is an important source of inhaled CO, both from mainstream and sidestream smoke (approximately 400 to 1,000 ppm) and from environmental tobacco smoke (1 to 18 ppm) (Wald and Howard 1975; National Research Council 1977; Turino 1981; Spengler 1991).

Carbon monoxide produces adverse health effects by interfering with the normal oxygen-carrying function of the blood. Because hemoglobin's affinity for CO is approximately 240 times greater than its affinity for oxygen, inhaled CO binds strongly, but reversibly, to hemoglobin to form carboxyhemoglobin (COHb). As a result of this competitive binding, and because hemoglobin releases oxygen more slowly in the presence of CO, the ability of the red blood cells to deliver oxygen to tissues is reduced. The resulting tissue hypoxia (lack of oxygen) may cause transient or permanent tissue damage, especially in those organs that demand high oxygen delivery, such as the brain and heart.

Blood COHb concentration serves as a biological measure of the integrated internal dose of CO to which a person recently has been exposed and is an excellent biomarker for CO exposure. Venous blood COHb levels are influenced by a number of factors: the CO concentration in the atmosphere, the duration of exposure, and certain physiological variables, especially pulmonary ventilation (Coburn et al. 1965). Nonsmokers with little or no environmental CO exposure have background levels of venous blood COHb between 0.5% and 1.0% due to the endogenous production of CO during normal metabolism (Coburn et al. 1965; Urbanetti 1981; Radford and Drizd 1982). Inhaling CO at the current NAAQS of 35 ppm (one-hour average) produces approximately 2% COHb in nonsmoking male subjects who exercise moderately for one hour (U.S. Environmental Protection Agency 1984). Smokers and workers in certain occupational settings typically have elevated blood COHb levels that range between 3% and 8% (Radford and Drizd 1982).

Although the toxic consequences of COHb levels in excess of 10% are well established, there has been considerable concern that small elevations in blood COHb may lead to adverse effects, especially in individuals with cardiovascular disease (Turino 1981; Rosenman 1990; Coultas and Lambert 1991). The possibility that there may be a link between CO exposure and cardiovascular disease is an important public health issue because of the widespread exposure to CO and the size of the population at risk.

CARDIOVASCULAR EFFECTS OF CARBON MONOXIDE

Despite a steady decline over the last two decades, cardiovascular disease is still the major cause of death in the United States (Gotto and Farmer 1988). Researchers have identified at least four risk factors for cardiovascular disease: family history; plasma cholesterol concentration; cigarette smoking; and high blood pressure. Recently, considerable attention has been focused on the possible association between other environmental factors, particularly CO exposure, and cardiovascular disease.

In considering the impact of CO exposure on the cardiovascular system, a distinction should be made between acute and chronic effects. Acute, or short-term, exposures to CO are a potential threat because inhaled CO can exacerbate myocardial ischemia (deficient blood flow to part of the heart muscle) in individuals with coronary artery disease, who already have limited ability to deliver oxygen to the heart. As has been suggested by some researchers, CO exposure may also increase the likelihood of fatal cardiac arrhythmias for these individuals. In contrast, prolonged or chronic exposure to low levels of CO might conceivably contribute to deaths from heart attacks by damaging the lining of blood vessels, thereby accelerating the development of atherosclerosis. This section will provide a brief review of the acute effects of CO exposure. It will focus on chronic CO exposures and the role of CO in the pathogenesis of heart diseases such as atherosclerosis.

Atherosclerosis is a progressive disease characterized by thickening of the arterial wall, and, in its advanced state, the development of atherosclerotic lesions in the large- and medium-sized arteries. The disease, which begins in childhood, involves several different biological processes. These include the proliferation of smooth muscle cells in a region of the artery called the intima (an inner lining of the blood vessel that is covered by a single layer of endothelium), the formation of connective tissue matrix by these proliferating smooth muscle cells, and the accumulation of cholesterol and other lipids (reviewed by Ross 1986, 1988; Hajjar and Pomerantz 1992). The advanced atherosclerotic lesion, called a fibrous plaque, consists of smooth muscle cells,

macrophages, connective tissue material, and varying amounts of lipids. As these plaques grow, they bulge into the lumen of the blood vessel, leading ultimately to complete occlusion of the artery.

The development of atherosclerosis is a complex and incompletely understood process. It appears to be initiated by an injury to the endothelium, the cell layer that lines the arteries. A number of factors have been postulated to cause the initial injury, including viruses, immunologic factors, and environmental toxins. As a result, serum factors and other cells are attracted to the damaged region, stimulating the replication of smooth muscle cells. Studies in animals show that the process can be accelerated by diets high in cholesterol or fat, and by environmental agents, such as chemical carcinogens, that stimulate cell proliferation (Ross 1986, 1988).

Acute Effects of Carbon Monoxide

The effects of acute, short-term CO exposures, leading to small elevations in blood COHb, have been studied in people with coronary artery disease. Most studies have been carried out in exposure chambers in which the subjects were exposed to defined levels of CO, both at rest and during exercise, and the resulting increases in COHb were carefully monitored. The most widely studied end points have been time to onset of angina pectoris (chest pain) and exercise performance. In selected studies, physiologic parameters (electrocardiography, angiography) and the frequency of arrhythmias have also been examined.

Studies in exercising subjects who have coronary artery disease have demonstrated that small increases in blood COHb levels limit exercise tolerance and shorten the time to the onset of angina. Although the results of some earlier studies (Aronow and Isbell 1973; Aronow et al. 1974) have been questioned, three independent groups of investigators recently reported a decrease in the time to onset of angina in exercising male subjects exposed to levels of CO sufficient to produce COHb levels in the range of 2% to 6% (Adams et al. 1988; Allred et al. 1989, 1991; The HEI Multicenter CO Study Team 1989; Kleinman et al. 1989). The HEI Multicenter CO Study Team (1989) also observed dose-related decreases in the length of time to onset of electrocardiographic ST-segment changes indicative of myocardial ischemia after CO exposure (COHb levels of 2.0% and 3.9%) (Allred et al. 1989, 1991; The HEI Multicenter Carbon Monoxide Study Team 1989).

Cardiac arrhythmias (abnormal heartbeats) are a complication of coronary artery disease and a major cause of heart attacks. One mechanism by which CO could contribute to sudden death in people with heart disease is by interrupting normal heart rhythm. There are, however, conflicting reports on the effects of CO on cardiac arrhythmias. One group (Chaitman et al. 1992) found no change in the frequency of ventricular arrhythmias at rest, during exercise, during recovery from exercise, or during usual activities when subjects with mild coronary artery disease and moderate levels of ventricular arrhythmias were exposed to CO sufficient to raise their blood COHb levels to approximately 3% or 5%. These results differ from those of Sheps and associates (1990, 1991), who reported a higher frequency of abnormal heartbeats during exercise in subjects with mild coronary artery disease and baseline arrhythmias, when blood COHb levels reached 6%. It is not known whether these conflicting results reflect differences in the study population, the study design, or problems associated with measuring small changes in a highly variable end point.

Ethical considerations limit experimental protocols in human subjects to investigations of relatively small increases in COHb in individuals with stable coronary artery disease. There are, however, several reports of associations between accidental exposures to moderate levels of CO in occupational settings (blood COHb levels of 10% to 40%) and sudden death from cardiac arrest (Balraj 1984; Atkins and Baker 1985; Rosenman 1990).

Chronic Effects of Carbon Monoxide

Whether or not chronic CO exposures are involved in the pathogenesis of progressive cardiovascular diseases, such as atherosclerosis, is an important public health concern because atherosclerosis is the leading contributor to deaths from heart attack and stroke in the United States (Myerburg and Castellanos 1988).

Interpretation of the few epidemiologic studies on the relation between chronic CO exposure and mortality from heart disease is limited by the absence of exposure information and the presence of potential confounders, such as smoking and other pollutants. Three studies have shown an association of morbidity (Kurt et al. 1978) or mortality (Cohen et al. 1969; Hexter and Goldsmith 1971) with heart disease and ambient CO concentrations. However, in an urban setting, Kuller and coworkers (1975) reported no relationship between environmental CO exposure and heart attacks.

Two occupational studies of the effect of exposure to motor vehicle exhaust indicate that chronic exposure to CO (and other pollutants) may be associated with an elevated risk of death from cardiovascular disease (Stern et al. 1980, 1988). In a study of New Jersey motor vehicle examiners, Stern and coworkers (1980) found that although there was no overall increase in deaths from cardiovascular disease in workers exposed to CO in the workplace, there was a two-fold increase of deaths classified as diseases of the arteries

and veins in workers exposed to CO when their death rates were compared to natural mortality rates ($p<0.05$). Possible sources of bias in this study include the lack of smoking information for the occupational cohort and the use of national mortality rates as a standard, instead of local rates. In a second report, which was a retrospective study of bridge and tunnel officers in the New York City Triborough system, the standard mortality rate for deaths from arteriosclerotic heart disease was 1.35 times greater (90% confidence interval 1.09, 1.68; $p<0.05$) for former tunnel officers than for the New York City population (Stern et al. 1988). The risk for tunnel officers was also greater than that for bridge officers (relative risk = 1.54; $p<0.01$). Smoking histories and personal CO exposure data were not available. Historical monitoring reports indicated that during the period of observation (1952 to 1981), 24-hour average CO levels were about 50 ppm in the tunnels. Peak levels of CO frequently exceeded 100 ppm during rush hour.

There is some evidence that, under certain conditions, the development of atherosclerosis in laboratory animals is accelerated by exposure to CO. Astrup (1967) first reported that CO exposure augmented the development of atherosclerotic-like lesions in the aortas of rabbits that were being fed high-cholesterol diets. Since that time, there have been other reports that the combination of an atherogenic diet (a diet that promotes atherosclerosis) and chronic exposure to high concentrations of CO increases the severity of atherosclerosis in rabbits (Davies et al. 1976), squirrel monkeys (Webster et al. 1968), Macaque monkeys (Thomsen 1974), and pigeons (Armitage et al. 1976; Turner et al. 1979).

In all of the above studies in which an atherogenic effect of CO exposure was reported, the animals inhaled relatively high levels of CO, which lead to 15% to 20% COHb. They also were being fed diets that contained 1% or 2% cholesterol or a combination of cholesterol and fat. An early report of CO-induced atherosclerotic lesions in rabbits fed a normal laboratory diet (Kjeldsen et al. 1972) was later retracted when a larger, more rigorously controlled study demonstrated no effect of prolonged exposure to 200 ppm CO, or brief exposures to either 2,000 ppm or 4,000 ppm CO, on atherosclerosis in male rabbits fed a normal diet (Hugod et al. 1978). These findings are not dissimilar to those of Penn and coworkers (1983), who reported that although a 14-week exposure to cigarette smoke accelerated the development of atherosclerotic lesions in cockerels, exposure to CO at concentrations equivalent to the CO levels in the cigarette smoke (45 ppm) had no effect.

Avian species are attractive models to study the pathogenesis of atherosclerosis. Unlike the rabbit, chickens (Moss and Benditt 1970) and some strains of pigeons (Clarkson et al. 1959) develop spontaneous atherosclerotic lesions when fed normal diets. In these species, the morphologic features and the developmental stages of the atherosclerotic plaques resemble their human counterparts. In chickens, the aortic plaques are composed of subendothelial accumulations of smooth muscle cells, abundant collagen, and extracellular accumulations of basement membrane–like material (Moss and Benditt 1970). The development of plaques can be modified by diet (Pick et al. 1962), or treatment with diet carcinogens (Albert et al. 1977; Penn et al. 1981) or drugs (Day et al. 1977).

White Carneau pigeons have been used extensively for studies of the pathogenesis of atherosclerosis as well as for investigations of the effects of environmental agents on the development of the disease. Armitage and coworkers (1976) studied the effect of simultaneous exposure to CO (150 ppm leading to approximately 10% COHb) and atherogenic (1% cholesterol) diets on the severity of atherosclerotic lesions in White Carneau pigeons. Carbon monoxide exposure had no effect on animals fed a normal diet. The severity of plaques in the aorta and coronary arteries was enhanced after 52 weeks of CO exposure in birds fed high levels of cholesterol. However, after 84 weeks of exposure, there were no differences between the CO- and air-exposed groups. The researchers then examined the influence of different levels of dietary cholesterol and different levels of CO exposure levels (Turner et al. 1979). As expected, the incidence and severity of coronary atherosclerotic lesions were associated with increasing dietary cholesterol. They were also associated with CO exposure in birds fed 0.5% or 1%, but not 2%, dietary cholesterol. In animals fed 1% cholesterol diets, the percentage of the coronary arteries affected by atherosclerotic plaques increased from 13% in air-exposed animals to 23% in animals with 10% COHb, and 33% in those with 20% COHb.

As the above discussion indicates, exposure to high concentrations of CO, leading to COHb levels in excess of 10%, exacerbates the development of atherosclerotic plaques in laboratory animals fed diets containing cholesterol. There is no evidence that CO has a similar effect on animals not subjected to the stress of an atherogenic diet or on animals with blood COHb values less than 10%.

JUSTIFICATION FOR THE STUDY

Because atherosclerotic heart disease is the leading cause of death in the United States, and because of the ubiquitous nature of CO emissions, investigation of the potential atherogenic action of CO is a high priority. The investigator's hypothesis was that "daily exposure to CO, at levels normally encountered in urban traffic patterns, is, by itself, not atherogenic, but can be atherogenic in animals with enhanced susceptibility to atherosclerosis." Dr. Penn aimed to

take advantage of a valuable model of atherosclerosis that he had developed in cockerels (Penn et al. 1980). These birds develop a naturally occurring atherosclerosis of their abdominal aorta with a fibromuscular plaque that resembles the lesions observed in human coronary arteries (Moss and Benditt 1970). Dr. Penn and his colleagues previously had shown that atherosclerotic plaque development in cockerels can be markedly accelerated by treatment with an agent that stimulates cell proliferation, such as the chemical carcinogen 7,12 dimethylbenz[*a*]anthracene (DMBA) (Penn et al. 1981), or by cigarette smoke (Penn et al. 1983). Thus, the investigator had a model for atherosclerosis that was well-suited to studies of the effect of environmental agents, such as CO, on the development and progression of this disease.

OBJECTIVES

Dr. Penn listed three major objectives in his original application:

1. to determine the atherogenic potential of different concentrations of CO in cockerels.
2. to determine whether exposure to a subatherogenic concentration of CO (as determined under Objective 1) promotes the development of atherosclerotic lesions in cockerels when combined with a diet that was mildly elevated in cholesterol. (The cholesterol content of the diet was to be manipulated in the presence and absence of CO, with animals exposed for 6, 12, or 18 months.)
3. to determine whether exposure of the animals to a subatherogenic concentration of CO prevents the regression of existing atherosclerotic plaques.

STUDY DESIGN

Because of technical difficulties, not all of the above objectives were addressed. The final study design explored CO as an atherogen, as a coatherogen, and as a modifier of preexisting plaques.

In studying CO as an atherogen, the investigator exposed cockerels (six or seven animals per group) to either air or CO (50, 100, 150, and 200 ppm) for two hours per day, five days per week, for 16 weeks.

In experiments focusing on CO as a coatherogen, cockerels (eight or nine animals per group) were fed either a standard diet or a diet supplemented with a low level of cholesterol (0.1%) from 6 to 22 weeks of age. During that time (16 weeks), the animals were exposed to either filtered air or 100 ppm CO daily for two hours per day, five days per week.

For experiments focusing on CO as a modifier of preexisting plaques, atherogenic plaques were stimulated in the cockerels during the period 6 to 22 weeks of age by either dietary supplementation with a high level of cholesterol (1.0%) or injection with subtumorigenic doses of the carcinogen DMBA. After completion of this protocol, the animals were exposed either to air or to 100 ppm CO for two hours per day, five days per week, for either 12 (DMBA) or 22 (cholesterol) weeks, to test whether CO stimulated further development of the lesions or prevented their regression.

In all experiments, the primary end point was the size of the atherosclerotic plaques, as measured by morphometric techniques, in the distal portion of the abdominal aorta.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

The questions asked by the investigator were posed in terms of whether the inhalation of specific levels of CO for particular time periods was sufficient by itself to augment arteriosclerotic plaque development, to enhance further the effect of known simulators of plaque development (cholesterol or DMBA), or to alter plaque development following the cessation of exposure to plaque-promoting agents. In each case, the investigator attained his immediate objective of exposing cockerels to defined time periods and levels of CO and to the desired plaque-promoting regimens. However, the chronic CO exposures for the coatherogen experiments were 16 weeks, rather than 6, 12, and 18 months, as originally planned. Dr. Penn attained his objective of accurately measuring plaque size, and in fact enhanced accuracy in this regard by adopting improved morphometric procedures during the course of the study. The investigator achieved his targeted CO levels within the exposure chamber with relatively narrow variance. However, he was unable to obtain reliable measurements of blood COHb levels in cockerels, a parameter that is needed to define CO dose and to relate the findings to humans.

METHODS

Dr. Penn was not able to detect any COHb in blood samples from the CO-exposed cockerels in the main set of experiments (Table 5 and Table 6). There were two fundamental problems with the investigator's approach to measuring blood COHb in cockerels, one being methodological and the other relating to the differences in the uptake and excretion of CO between avian and mammalian species.

For his determinations of cockerel blood COHb, Dr. Penn

employed the same CO-oximetry method that is used to measure COHb in humans. CO-oximetry, a standard spectrophotometric technique for measuring CO levels in blood, is based on the changes in the absorption spectrum brought about by the combination of CO with hemoglobin. Different types of hemoglobin have different absorption spectra for COHb. It is unlikely that cockerel hemoglobin would have spectrophotometric qualities similar to those of human hemoglobin. To make this analysis work would require careful study of cockerel COHb in vitro, including plotting the isobestic points and evaluating the interference from methemoglobin and other hemoglobin subtypes. The finding of high baseline blood COHb levels in a subgroup of cockerels examined after the main study had been completed (Table 8) calls into question the accuracy of the CO-oximetry procedures.

A second problem, and one which the investigator tried to address at the end of the study, was the kinetics of CO uptake and elimination in the experimental model. From what we know about the respiratory system of birds, one can predict that uptake and elimination of CO in the cockerel will be rapid. However, standard procedures, such as the Coburn-Forster-Kane model (Coburn et al. 1965) can not be used to predict COHb levels without information about the affinity ratio or the equilibrium constant for the displacement of oxyhemoglobin by CO.

The major end point for study was plaque volume, as determined by morphometry. (The originally planned labeling studies, which were to complement the morphometric studies, were not performed.) The precise and objective morphometric procedures used for quantifying atherosclerosis are a strength of the study. The investigator obtained 5-mm sections of each aorta from the iliac arteries to the thoracic aorta. Dr. Penn adopted a new means of analysis of the volume of the plaques seen under the microscope. A modified point-counting procedure, using a grid superimposed on a projected magnification of the arterial section, allowed him to treat the entire section as a single unit, thereby minimizing some of the problems caused by fixing and further sectioning the aorta sample. Although this method of point counting is now widely accepted, many of the earlier studies reporting on the atherogenic effect of CO did not utilize such rigorous quantitative procedures for measuring plaque size.

STUDY DESIGN

For the atherogenesis experiments, Dr. Penn exposed the animals to either air or one of five concentrations of CO in air (ranging from 50 ppm to 200 ppm) for two hours per day, five days per week, for 16 weeks. Although exposure to these concentrations results in high CO body burdens in humans, such may not be the case in cockerels because birds have different rates of CO uptake and elimination. The limited information regarding the actual COHb levels achieved under the exposure regimen suggests that the increase in COHb, whatever it was, was present only during the time of exposure. Thus, comparisons with the human condition would necessitate longer exposures.

Dr. Penn chose 100 ppm CO rather than 200 ppm as the exposure level for the coatherogenicity study; however, the rationale for this choice is not convincing. The investigator stated that he chose 100 ppm because it was as ineffective as 200 ppm CO in promoting plaque development, and because 100 ppm is at the upper end of urban CO levels. However, this was not primarily a range-finding study, but one aimed at determining whether the model was of value in detecting an effect of CO on atherogenesis. Thus, the higher level would have been more appropriate, particularly in conjunction with a treatment that by itself did not cause a statistically significant increase in atherogenesis. Furthermore, as hypothesized by the author, if CO is released more rapidly from cockerel hemoglobin than from human hemoglobin, it is difficult to understand why assumptions about the relationship between study design and urban CO values were the main factors in selecting the CO exposure concentrations.

In the CO modification experiments, it is not clear why the investigator applied CO exposure after treatment with a high cholesterol diet or DMBA rather than combining CO exposure with the atherogenic treatments. Simultaneous exposures would have been more realistic in relation to human arteriosclerosis.

STATISTICAL METHODS

The authors used one-way analysis of variance to compare plaque volume percentages in groups of animals subjected to different regimens of exposure to carcinogens, cholesterol, and CO. The repeated measures analysis of variance was used to compare levels of COHb in cockerels at different intervals after removal from the exposure chamber. The measurement and statistical methods used in this study are sufficiently sound to support the investigator's analysis of the results.

RESULTS AND INTERPRETATIONS

No significant differences in plaque volume at any exposure level were observed in the first set of studies that evaluated the atherogenic potential of exposure to CO in cockerels (Table 1). In the coatherogen experiments using DMBA treatment or a 0.1% cholesterol diet, there were no statistically significant differences in any treatment group

with regard to the volume of the atherosclerotic plaques (Table 2). The investigator confirmed a previous observation that DMBA treatment produces an increase in the volume of atherosclerotic plaques in the abdominal aorta of cockerels 12 weeks after the cessation of treatment (Penn et al. 1981). A similar effect appeared to occur with a 1% cholesterol diet, although the data were not statistically significant. In both cases, subsequent exposure to CO produced a statistically nonsignificant decrease in plaque volume compared with air controls.

As part of the dietary study, the investigators evaluated blood cholesterol levels. Although there was clearly an observable effect of dietary treatment, CO exposure had no impact on serum cholesterol levels.

Dr. Penn found that exposing cockerels to CO under a number of defined conditions had no effect on the rate of development or regression of atherosclerotic plaques in the abdominal aorta. Unfortunately, the data permit interpretation of this study only in terms of exposure-response rather than dose-response. In other words, we can be reasonably comfortable in accepting the data on the levels of CO in the cockerel exposure chamber and the morphometric analysis of the extent of the response in terms of the size of the atherosclerotic lesions. However, we have no reliable evidence regarding the blood COHb levels that presumably would affect arteriosclerosis, and by which we would attempt to extrapolate the relevance of any negative or positive effect to humans. Although measuring the exposure-response relationship is not unusual for other air pollutants, it is a step backward for studies of CO, for which the ability to measure the amount of CO bound to hemoglobin has permitted quantifying internal dose and relating this parameter to effects.

If the author is correct in his conclusion that CO is released relatively rapidly from cockerel hemoglobin following the cessation of exposure, then the relatively short exposure periods of two hours per day for five days per week suggest that the animals' COHb levels were elevated for only minimal periods. Because the half-time for COHb reequilibration in humans is two to six hours (Peterson and Stewart 1970; Landaw 1973), humans with significant CO exposure maintain elevated COHb levels for much of the day. If, in fact, cockerel carboxyhemoglobinemia is maintained only during the exposure periods, then the maximum study periods (two hours daily for 16 weeks) used in this study are not representative of the chronic exposure scenario.

It is also possible to question the investigator's contentions that (1) the apparent rapid dissociation of cockerel COHb means that COHb most likely also forms very rapidly; and (2) the result of this was a steady-state COHb concentration for cockerels for 100 for 110 minutes of the exposure period. Although a rapid dissociation of COHb most likely indicates that there would be a rapid association, in addition to the physicochemical factors governing hemoglobin and CO interactions, respiratory rate and minute ventilation are also factors. The usual animal response to handling is a marked increase in ventilation. Presumably, this occurred while the animals were placed in the chamber, giving them time to settle back to more normal ventilation before peak chamber CO levels were achieved, and after they were taken from the chamber and prepared for blood sampling; this time period would affect the apparent rate of COHb disassociation. In summary, we know little about cockerel COHb, a problem that precludes giving much weight to this otherwise well-performed study.

For any study in which the findings are negative, it is important to discern to what extent the results respond to the study's objectives and lay to rest the questions being addressed. The central question underlying this study was whether CO exposure influences the development of atherosclerosis in humans. Because of a period of carboxyhemoglobinemia that can, at best, be described as apparently short, this study fell short of providing a test model suitable to investigate CO-induced arteriosclerosis. Accordingly, the findings provide little faith in the null hypothesis.

IMPLICATIONS FOR FUTURE RESEARCH

There is still no definitive answer to the question of whether exposure to CO is a risk factor for atherogenesis. The evidence favoring such a role for CO at ambient levels is minimal, but because of the importance of the problem, it warrants further evaluation. The present study makes it unlikely that the cockerel will serve as a suitable model for the study of CO-induced atherogenesis. However, even this can not be ruled out. Based upon the present data, it is clear that other investigators considering this model will need to evaluate carefully the interaction between CO and the oxygen-combining site of hemoglobin in cockerels. They also will need to consider much longer durations of exposure, and they should evaluate the coatherogenic effect of CO using a simultaneous atherogenic stimulus. The present study demonstrates the need for careful exploration of CO-hemoglobin interactions before beginning the study of CO in any animal model.

CONCLUSIONS

The potential for CO to initiate or accelerate the development of atherosclerosis was studied in a laboratory avian model. Cockerels develop spontaneous atherosclerotic le-

sions in their abdominal arteries. Because these lesions resemble atherosclerotic plaques in human coronary arteries and because their development can be modified by diet and carcinogens, such models are useful for studying the effects of environmental agents on the initiation and progression of atherosclerosis.

Dr. Penn evaluated whether inhalation of CO (50, 100, 150, and 200 ppm for two hours per day, five days per week, for 16 weeks) enhanced the spontaneous development of atherosclerotic plaques, had a coatherogenic effect when administered in combination with 0.1% cholesterol, or altered the development of preexisting plaques. No significant differences in the volume of atherosclerotic plaques in the abdominal aorta of the cockerels were observed at any CO exposure level in studies that examined either the atherogenic or coatherogenic potential of CO. As expected, treatment with the carcinogen DMBA resulted in an increase in the volume of atherosclerotic plaques, but subsequent exposure to CO had no further enhancing effect.

Carbon monoxide exposures were evaluated in terms of blood COHb levels. Unfortunately, because of methodological problems, reliable blood COHb levels were not obtained. Thus, in this study, there is no link between external CO exposure and internal dose. Because of the differences between avian species and mammals in their uptake and elimination of CO, it is possible that the actual CO body burden was relatively minimal and not comparable to the human situation.

Because of the uncertainties regarding the CO exposures in the cockerel, this study did not provide a suitable test model. Therefore, it is not possible to conclude from these results whether or not CO affects avian or human atherosclerosis.

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Research Report Number 57 May 1993

HEI HEALTH EFFECTS INSTITUTE

Statistical Methods for Epidemiologic Studies of the Health Effects of Air Pollution

William Navidi, Duncan Thomas, Bryan Langholz, and Daniel Stram
University of Southern California School of Medicine, Los Angeles, CA

Includes the Commentary of the Institute's Health Review Committee

Research Report Number 86
May 1999

HEI HEALTH EFFECTS INSTITUTE

The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI studies all major pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate matter), and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 200 projects at institutions in North America and Europe.

Typically, HEI receives half its funds from the U.S. Environmental Protection Agency and half from 28 manufacturers and marketers of motor vehicles and engines in the United States. Occasionally, funds from other public or private organizations either support special projects or provide resources for a portion of an HEI study. Regardless of funding sources, HEI exercises complete autonomy in setting its research priorities and in reaching its conclusions. An independent Board of Directors governs HEI. The Institute's Research and Review Committees serve complementary scientific purposes and draw distinguished scientists as members. The results of HEI-funded studies are made available as Research Reports, which contain both the Investigators' Report and the Review Committee's evaluation of the work's scientific quality and regulatory relevance.

HEI Statement

Synopsis of Research Report Number 86

Statistical Methods for Epidemiologic Studies of the Health Effects of Air Pollution

BACKGROUND

Using epidemiologic approaches to determine the health risks of exposure to air pollutants is challenging: it is difficult to measure exposure to the relatively low levels of pollutants to which people are generally exposed, and to find populations with different degrees of pollutant exposure but comparable exposure to potentially confounding factors. Furthermore, the frequently high correlation among different pollutants makes it difficult to identify the effect of a single agent. In the early 1990s the Health Effects Institute (HEI) supported an Environmental Epidemiology Planning Project to identify the methodological issues that needed to be addressed in future studies of the health effects of air pollutants (HEI Communications Number 3, 1994). HEI also funded research targeted to the development of epidemiologic methods in this area of air pollution research and, when possible, to the testing of these methods using appropriate data sets. HEI funded the study conducted by Dr. William Navidi and colleagues to develop statistical methods that would improve the estimates of the health effects air pollution obtained from epidemiologic studies. One important feature of this study was that the investigators were able to test the statistical models they developed using data from the University of Southern California Children's Health Study of the long-term effects of air pollutants on children.

STATISTICAL METHODS AND CRITIQUE

In this report, Navidi and colleagues discussed the development of three sophisticated statistical methods and their application to air pollution epidemiologic studies. First, they took a standard case-crossover design (an approach to assessing the effect of transient exposures on the risk of onset of an acute event) and introduced a bidirectional element where control data were obtained both before and after the health event of interest. Navidi and colleagues showed that their bidirectional case-crossover design method provided a better estimate than the standard case-crossover design and illustrated the potential bias in the latter approach. The use of the bidirectional case-crossover promises to be an advance over the use of the case-crossover design and offers promise for reducing bias in environmental epidemiologic studies. More investigation is needed, however, into possible problems, such as sensitivity to the mathematical model employed and "carryover effects"—the individual behaving differently after the health event.

Second, because measurement error (the difference between true and measured exposures) can have a substantial impact on the accuracy of estimated health effects, Navidi constructed a model to evaluate the reliability of two approaches to estimating cumulative exposure to air pollutants. The investigators imply that neither the direct nor the indirect approach to measuring exposure gave reliable answers when uncorrected measurement errors were present. This conclusion appears justified based on the simple model the investigators used. The investigators believe that more elaborate models may correct themselves and thus result in reliable estimates of cumulative exposure, but it is not clear how this will occur. Additional modeling and simulations are needed to make any definite conclusions. Certainly more basic research is needed to understand if the two approaches can be shown capable of estimating cumulative exposures and, if so, how well.

Third, the investigators adapted a multilevel analytic design to air pollution epidemiology. This design has the potential to combine individual and group level comparisons. Such designs, which have been used for many years in the social sciences and are now being applied in health effects fields, are undergoing intense development. Navidi has made an advance in this area by identifying the components needed to model the health effects of air pollution and by developing reasonable mathematical models. Further work is needed to determine the sensitivity of the investigators' multilevel model to model assumptions and bias before it can be recommended for general use.

COMMENTS

Navidi and his collaborators have made advances in statistical methodology. Their main contribution has been less in the development of new methods than in applying and extending methods in use in other areas—particularly the bidirectional case-crossover design and multilevel analytic design—to the air pollution health effects field. Some issues remain unresolved; applications of the investigators' methods to exposure problems that include careful model development and validation are needed to resolve these questions.

This Statement, prepared by the Health Effects Institute and approved by its Board of Directors, is a summary of a research project sponsored by HEI from March 1993. This study was conducted by Drs. William Navidi, Duncan Thomas, Bryan Langholz, and Daniel Stram of the University of Southern California, Los Angeles, CA. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

Library of Congress Catalog Number for the HEI Research Report Series: WA 754 R432.
The paper in this publication meets the minimum standard requirements of the ANSI Standard Z39.48-1984 (Permanence of Paper) effective with Report Number 21, December 1988, and with Report Numbers 25, 26, 32, 51, and 65 Parts IV, VIII, and IX excepted. These excepted Reports are printed on acid-free coated paper.

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INVESTIGATORS' REPORT

Statistical Methods for Epidemiologic Studies of the Health Effects of Air Pollution

William Navidi, Duncan Thomas, Bryan Langholz, and Daniel Stram

This Investigators' Report was produced with a different page layout than other Research Reports because of the incompatibility of the program used to create the equations with our desktop publishing software. To prevent errors being introduced in the process of recreating equations and to ensure that the mathematical characters set down by the authors maintained their precision and authenticity, we used camera-ready copy produced by the authors and had them stripped into the pages by our printer.

The Abstract begins on page 2.

* A list of abbreviations appears at the end of the Investigators' Report.

This Investigators' Report is one part of Health Effects Institute Research Report Number 86, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. William Navidi, Colorado School of Mines, Department of Mathematical and Computer Science, Golden, CO 80401.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award R824835 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement by it should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

0 Abstract

We describe two statistical designs that can provide efficient estimates of the health effects of exposure to air pollutants in epidemiologic studies. We also evaluate the effects of measurement error in exposure assessment on the accuracy of estimated health effects.

The bidirectional case-crossover design is a variant of a method proposed by Maclure (1991). Our version of the method takes advantage of the fact that in epidemiologic studies involving environmental exposure, accurate information about past exposure is more readily available, and that levels of exposure are generally unaffected by the response of the subject. It differs from other case-crossover methods in that control information is assessed both before and after failure, thus avoiding confounding due to time trends in exposure.

The multilevel analytic design provides a method of combining estimates of health effects made on the individual level with those made at the group level. It has great potential value in situations where variations in exposure within groups may not be great enough to provide adequate power to detect health effects, as is often the case in air pollution studies where exposure levels are similar within a geographic community.

Measurement errors in exposure assessment can have substantial impact on the accuracy of estimated health effects. When the microenvironmental approach is used to estimate exposure, a standard error of 30% in estimating indoor/outdoor ratios can increase the standard error of a relative risk estimate by 50%, and introduce bias as well. Similar results hold when exposure is estimated with personal samplers. When the microenvironmental approach is used, errors in estimating indoor/outdoor ratios have more influence on the accuracy of risk estimation than do errors in estimating the time spent in microenvironments.

1 Introduction

Fundamental challenges common to many environmental epidemiological studies include (1) difficulties in measuring exposure, disease, and related variables, (2) the problem of finding populations with different degrees of exposure that are comparable with respect to potentially confounding factors, and (3) the difficulty of distinguishing effects at the individual level, often as a result of the spatially clustered nature of many environmental exposures.

We have addressed the methodological implications of these challenges in several ways. We studied an epidemiologic design that combines two approaches to studying exposure-disease associations, the analytic approach based on comparisons between individuals (for example, case-control and cohort studies), and the ecologic approach based on comparisons between groups. The analytic approach generally provides a stronger basis

for inference, in part because of freedom from between-group confounding and better quality data, but the ecologic approach is less susceptible to attenuation bias from measurement error and may provide greater variability in exposure. The design we propose entails the selection of a number of groups and enrollment of individuals within each group. Exposures, outcomes, confounders, and modifiers are to be assessed on each individual, but additional exposure data might be available on the groups. The analysis then combines both the individual-level and the group-level comparisons, with appropriate adjustments for exposure measurement errors, and tests for compatibility between the two levels of analysis, for example to determine whether the associations at the individual level can account for the differences in disease rates between groups. We also studied the implications for design efficiency in terms of trade-offs between numbers of groups, numbers of individuals, and the extent of the individual and group measurement protocols.

We have studied the impact of measurement error in assessing individual exposure to air pollutants on the accuracy of the estimation of health effects. We have considered the "microenvironmental" approach, the personal sampler (badge) approach, and an approach in which each individual's exposure was estimated to be the ambient outdoor level measured at a monitoring station. In the microenvironmental approach, the region occupied by an individual during the time period of interest is divided into a number of "microenvironments", e.g. home, school, office, car, outdoor areas, etc. To estimate an individual's cumulative exposure one estimates the concentrations of the pollutant of interest and the times spent by the individual in each microenvironment, then computes the appropriate time-weighted average. In the personal sampler approach, each individual wears a badge that traps or reacts with the pollutant to provide an estimate of the cumulative exposure. We have gained considerable insight into the association between measurement error in exposure assessment and bias in the estimation of health effects. This should be useful in the development of validation studies whose results can be used to improve the accuracy of these estimates.

We have also studied the case-crossover design (Maclure, 1991) for examining the acute effects of exposure to air pollutants. This is a design in which only cases are sampled, and their exposure at the time of their failure is compared with some estimate of their typical level of exposure. The definition of "failure" varies from study to study. For example, in the USC Children's Health Study, failure is defined as a day of absence from school. In studies of the relationship between particulate exposure and mortality, failure refers to death. Mittleman, Maclure, and Robins (1995) discuss Maclure's method and some variations on it. In all of the methods they describe, all control information is assessed prior to failure. Sampling all control information prior to failure makes the case-crossover design subject to bias from time trends in exposure. While time trends in exposure can be quite pronounced for environmental exposures, studying the effects of environmental exposures offers two compensating advantages. First, accurate information is often available about the levels of past exposure. Second, exposure after an event is unaffected by the occurrence of the event. We describe a modification of the case-crossover approach that makes use of both of these advantages. Our approach involves assessing control

information both before and after failure, which avoids confounding due to time trends.

We have applied some of these ideas to the University of Southern California Children's Health Study. This is an ongoing study of the health effects of air pollution in Southern California, in which 12 communities with different levels and types of pollution have been selected and more than 3500 school children have been enrolled in a ten-year cohort study. In this study, exposure assessment protocols involve a combination of ambient monitoring, microenvironmental sampling, personal sampler monitoring, and questionnaire data on time-activity and household characteristics. Outcomes of interest include several measurements of pulmonary function, as well as morbidity outcomes as reported on questionnaires and as indicated by school absences.

2 Specific Aims

This study had three specific aims: (1) to develop efficient statistical designs for panel studies, (2) to develop methods for allowing for the effects of exposure measurement error, and (3) to develop methods for combining individual and aggregate level comparisons.

Our work on the bidirectional case-crossover design addressed the first aim by presenting a statistical design that has proven successful in analyzing acute health effects of exposure to air pollutants in a major epidemiologic study. We also addressed this aim in our work on the multilevel analytic design, by measuring the impact of design decisions involving trade-offs between numbers of groups, numbers of individuals, and the extent of the individual and group measurement protocols.

We addressed the second aim in two ways. We developed mathematical models for measurement error that allow us to compare the accuracy of the indirect or microenvironmental approach to exposure assessment with the direct or personal sampling approach. We also assessed the impact of these errors on a model that describes the effect of exposure on a health outcome.

We addressed the third aim by developing the multilevel analytic design. A basic feature of this design is that it can be applied at the individual level, by studying the within-group differences, or at the aggregate level, by studying the group averages. The within-groups and the between-groups estimates can be combined to yield a pooled estimator that is more efficient than either of the two estimates separately.

3 The Bidirectional Case-crossover Approach

3.1 Introduction

The case-crossover design (Maclure, 1991) is a design in which only cases are sampled, and their exposure at the time of their failure is compared with some estimate of their typical level of exposure. This design has the advantage that by comparing exposures within subjects, all time invariant confounders such as sex, race, diet, age (over short periods), and community of residence are automatically controlled for.

Data analysis in case-crossover studies is done by standard case-control methods. The basic principle is to estimate risk by comparing the exposure of the subject during a time interval just before failure (the case interval) with the exposure during one or more prior time periods (control intervals). Conditional logistic regression is generally used, with the Mantel-Haentzel approximation an option when the exposure measurement is binary.

In crossover experiments, it is common to randomize the order of treatment, so that in a clinical trial, for example, approximately equal numbers of subjects receive treatment followed by placebo as the other way around. Randomization prevents the order of treatment from being confounded with other risk factors. In observational studies this is of course impossible. The fixed sequence of exposures, combined with the fact that the case exposure is always sampled last, makes the case-crossover design subject to bias from time trends in exposure. Greenland (1996) points out that this is a form of selection bias, since it tends to cause controls to be systematically either more or less exposed than cases. In fact, it is the same sort of bias that could occur in a standard case-control study if the controls were selected earlier in time than the cases.

A number of variations of the case-crossover design have been proposed, involving different strategies for selecting control information. Mittleman, Maclure, and Robins (1995) describe methods in which one or more control intervals are selected at specified time lags before the event, which provide analogs to a one-to-one or many-to-one matched case-control study. Alternatively, one may estimate the proportion of time exposed during a longer interval (e.g., one year) before the event. This is the approach used by Maclure (1991), and referred to by Mittleman, Maclure, and Robins as the "Usual Frequency Approach." Marshall and Jackson (1993) describe a version of this approach in which exposure could be continuous rather than binary. In all of these approaches, secular trends in exposure are assumed to be absent, although Mittleman, Maclure, and Robins (1995) describe several methods for adjusting for cyclical trends. Feldman (1993ab) describes a method for estimating acute and transient risks associated with intermittent exposure. It is designed for binary exposure and for non-terminal outcomes (i.e., outcomes other than death). It does not automatically adjust for individual susceptibility factors, but instead requires that baseline risk be correctly modeled as a function of measured covariates.

Time trends can be particularly strong for environmental exposures such as air pollutants.

Thus standard case-crossover methods can be severely biased. However, studying the effects of environmental rather than behavioral exposures offers two advantages. First, fairly accurate information about past levels of exposure is often available. Second, levels of exposure are unaffected by failures of subjects. Therefore it is possible to determine at times post-failure what the level of exposure would have been had a subject not failed. The case-crossover approach can be adapted to make use of these advantages by assessing control information from times both before and after failure. We refer to this method as the *bidirectional* case-crossover method, since control information is assessed in both directions from the failure time. This allows consistent estimators of risk to be computed, regardless of time trends in exposure.

3.2 The Case-crossover Design for Environmental Exposure

3.2.1 Methods for School Absence Data

We have applied the bidirectional case-crossover design to study the relationship between school absence and pollution levels in the USC Children's Health Study (Peters, 1996). We describe an approach that is appropriate for this situation. For each subject i, let $t_1, ..., t_M$ be the set of days at risk, that is, the set of days that school is in session for that subject, and let X_{ij} be the exposure of subject i on day t_j. The quantity X_{ij} may be multivariate, containing levels of several pollutants at several lags, as well as values of potential confounders. Assume the log odds of absence for subject i on day t_j are given by

$$\log \frac{p_{ij}}{1 - p_{ij}} = \lambda_i + \beta X_{ij}. \tag{1}$$

where λ_i is the baseline level specific to subject i. Then

$$p_{ij} = \frac{e^{\lambda_i} e^{\beta X_{ij}}}{1 + e^{\lambda_i} e^{\beta X_{ij}}}. \tag{2}$$

We assume for simplicity that absences on distinct days are independent. While this is not strictly true, it is nearly so for days far apart in time. With a reasonably large amount of data, this assumption should be satisfactory. Assume that subject i is absent on exactly n_i days, and let A_i denote the set of days on which he is absent. It follows that the probability that a subject is absent precisely on these n_i days is

$$\mathrm{P}(A_i) = \left(\prod_{t_j \in A_i} p_{ij} \right) \left(\prod_{t_k \notin A_i} (1 - p_{ik}) \right). \tag{3}$$

Conditional on the number of absences being n_i, the probability that the absences occurred

precisely on those days in A_i is

$$\mathrm{P}(A_i|n_i) = \frac{\left(\prod_{t_j \in A_i} p_{ij}\right)\left(\prod_{t_k \notin A_i}(1-p_{ik})\right)}{\sum_{S \in D_{n_i}}\left(\prod_{t_j \in S} p_{ij}\right)\left(\prod_{t_k \notin S}(1-p_{ik})\right)}, \tag{4}$$

where D_{n_i} denotes the collection of all sets of n_i days. Substituting (2) into (4) yields

$$\mathrm{P}(A_i|n_i) = \frac{e^{\beta \sum_{t_j \in A_i} X_{ij}}}{\sum_{S \in D_{n_i}} e^{\beta \sum_{t_k \in S} X_{ik}}}. \tag{5}$$

Let $X_{A_i} = \sum_{t_j \in A_i} X_{ij}$, and for each subject i and each set of n_i days S, let $G_{iS} = \sum_{t_k \in S} X_{ik}$.

Then

$$\mathrm{P}(A_i|n_i) = \frac{e^{\beta X_{A_i}}}{\sum_{S \in D_{n_i}} e^{\beta G_{iS}}}. \tag{6}$$

The likelihood function is found by summing the logarithm of (6) over all subjects:

$$L(\beta) = \sum_i \left[\beta X_{A_i} - \log \sum_{S \in D_{n_i}} e^{\beta G_{iS}}\right]. \tag{7}$$

We estimate β by maximizing the right hand side of (7). The Fisher information is the negative of the second derivative of (7). In the case where β is univariate, this is given by

$$I(\beta) = \sum_i \frac{(\sum_{S \in D_{n_i}} G_{iS}^2\, e^{\beta X_{iS}})(\sum_{S \in D_{n_i}} e^{\beta X_{iS}}) - (\sum_{S \in D_{n_i}} G_{iS}\, e^{\beta X_{iS}})^2}{(\sum_{S \in D_{n_i}} e^{\beta X_{iS}})^2}. \tag{8}$$

The variance of the maximum likelihood estimate of β ($\hat{\beta}$) is estimated by substituting $\hat{\beta}$ for β in (8) and inverting.

The number of sets to be summed over in the denominator of (5) is $\frac{M!}{n_i!(M-n_i)!}$, where M is the total number of days at risk. This is too large for the sum to be computable. In practice, therefore, we select a few sets at random and sum over these. Results of Langholz and Goldstein (1997) imply that under this approach, equations (5)–(8) remain valid, with sums over the full collection of sets D_{n_i} replaced by sums over the randomly chosen subcollection of sets.

3.2.2 Methods for Mortality Data

In the case of mortality data, the probability that subject i fails at time t_j is given by the proportional hazards model

$$\Lambda_i(t_j) = \lambda_i \exp(\beta X_{ij}). \tag{9}$$

Conditional on having failed during the ascertainment period, the probability that subject i fails at time t_k is

$$p_{ik} = \frac{\exp(\beta X_{ik})}{\sum_{j=1}^{M} \exp(\beta X_{ij})}. \tag{10}$$

The log likelihood function can be derived in a manner analogous to that of the previous section. If the failure time of the ith subject is t_{k_i}, the log likelihood function is

$$L(\beta) = \sum_{i=1}^{n} \log p_{ik} = \sum_{i=1}^{n} \left[\beta X_{ik_i} - \left(\log \sum_{j=1}^{M} \exp(\beta X_{ij}) \right) \right]. \tag{11}$$

3.3 Simulations

We have conducted two simulation studies to evaluate the accuracy of this approach. In the first study, 1000 subjects were followed for 100 days. A univariate exposure X was generated, taking values in the repeated pattern $1, 1.5, 2, 2.5, 3, \ldots$. The true value β of the log relative risk associated with one unit of exposure was taken to be 1, and the baseline e^{λ} was taken to be 0.005 for each subject. In this way the mean number of days absent for each subject was 4.69. Table 1 presents the results. The column labeled "Number of Combinations" gives the number of sets of n_i days used to form sum in the denominator of (5). The column labeled "Mean" gives the average of the 1000 estimates of β, and the column labeled "SD" gives the standard deviation for that sample, which should be quite close to the standard error (SE) of $\hat{\beta}$. To assess the accuracy of the Fisher information estimate of the SE, The column labeled "Nominal SE" (Root Mean Square Standard Error) gives the square root of the average of the 1000 variance estimates computed by inverting the Fisher information number.

The results show that there is no appreciable bias, even when the denominator in (5) is estimated with a single control set. The standard deviation is reduced by increasing the number of control sets, but it is well estimated with the standard Fisher information estimate in all cases.

Our second simulation used actual exposure data collected for the Children's Health Study. We obtained data on concentrations of particulate matter 10 microns or less in diameter

Table 1: Simulation Results for the Bidirectional Case-crossover Design

True Value	Number of Combinations	Mean	SD	Nominal SE
1.000	1	1.006	0.0646	0.0635
1.000	10	1.001	0.0331	0.0332
1.000	100	1.001	0.0261	0.0266

(PM_{10}) for ten Southern California communities for each day from January 1 through December 30, 1994. (Several of the communities had some days with missing information; these days were ignored in the simulation.) For purposes of the simulation, we assumed there were 100,000 subjects at risk in each community on January 1. The probability that a subject in community i would fail on day j was given by (9). The exposure X_{ij} was taken to be the average daily concentration of PM_{10}, in units of $100\mu g/m^3$. We took the baseline hazard λ_i to be 10^{-5} for all subjects in all communities, and the true value of the log odds ratio β to be 0.1. This results in a true relative risk of about 1.1 per $100\mu g/m^3$, which is in good agreement with relative risks of mortality due to particulates as estimated by Schwartz (1994) and Schwartz and Dockery (1992).

Figure 1 shows a plot of the daily average levels of PM_{10} for Mira Loma, California, one of the ten communities. The time trends in this town are representative of most of the others, and of particle pollution in Southern California generally. Levels are highest in the winter and in the summer. The winter peak is related to cloud cover, stagnation, and primary emissions, while the summer peak is a result of photochemistry and emissions.

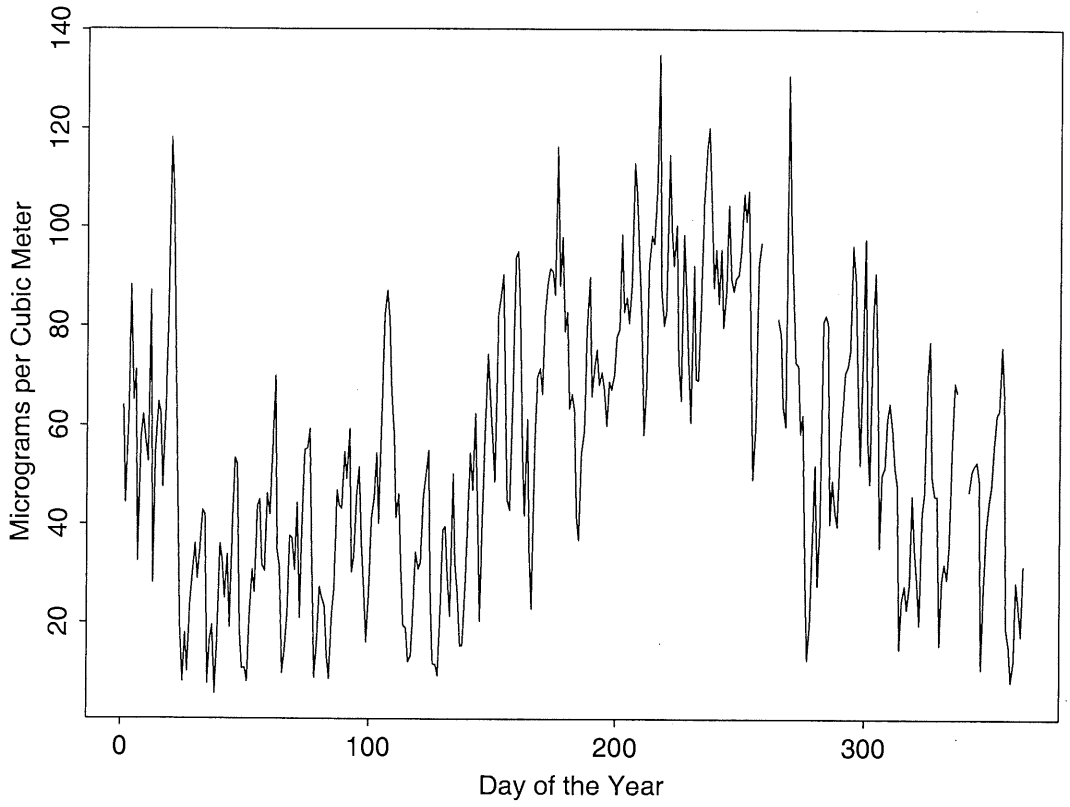

Figure 1: PM_{10} levels in Mira Loma, California, 1994.

We generated 1000 artificial data sets. Each data set was constructed by generating, for each community i and each day j, an observation from a Poisson distribution with mean $N_{ij}\lambda_i e^{\beta X_{ij}}$ where N_{ij} represents the number of subjects at risk in community i on the jth day. For each data set, we computed five different estimates of β. Two of them, $\hat{\beta}_{lag14}$ and $\hat{\beta}_{lag28}$, are conventional case-crossover estimates, each using a single control day for each subject 14 or 28 days before failure, respectively. $\hat{\beta}_{1-1}$ is the bidirectional case-crossover estimate with a single control day for each subject randomly chosen from among the 363 control days. $\hat{\beta}_{TH}$ is a "total history" version of the case-crossover estimator, in which all days prior to failure are used as controls. Finally, $\hat{\beta}_{BI}$ is the bidirectional case-crossover estimator using the full stratum of 363 control days for each subject. For each data set, we computed each of the five estimators of β along with its estimated asymptotic variance. Thus we had 1000 replications of each estimator, along with 1000 replications of its estimated asymptotic variance.

The third column of Table 2 presents, for each estimator, the average of the 1000 replications across the simulated data sets. It can be seen that $\hat{\beta}_{1-1}$ and $\hat{\beta}_{BI}$, the two bidirectional case-crossover estimators, are nearly unbiased, while the others, which use only information prior to failure, are considerably biased. The fourth column presents for each estimator the sample standard deviation of the 1000 replications, which should be a good approximation to the standard error. As expected, estimators using more control information have smaller standard errors. In fact, the standard error of $\hat{\beta}_{1-1}$ is greater than that of $\hat{\beta}_{BI}$ by a factor very close to $\sqrt{2}$, which is in accordance with the rule of thumb for case-control studies that the relative efficiency of a case-control study with M controls per case is $M/(M+1)$ compared to a cohort study. The fifth column of Table 2 presents, for each estimator, the square root of the average of the 1000 replications of its asymptotic variance. The estimated asymptotic variance is seen to be nearly unbiased for the true variance for each of the estimators.

We can obtain some insight into the nature of the bias for $\hat{\beta}_{lag14}$ and $\hat{\beta}_{lag28}$ by examining the time trends in the data more closely. For the lag 14 data, only those cases who fail later than January 14 are considered in the analysis. Their exposures at failure are compared with their exposures 14 days earlier. In all 10 communities taken together, 50.1% of the days after January 14 had lower levels than their corresponding control day. On average, exposure on days after January 14 was 0.08 $\mu g/m^3$ lower than on the corresponding control day. Thus there is a slight tendency for control days to have greater exposure. When applied to the 1,000,000 subjects in the simulation, it causes the effect of exposure to be underestimated, as shown in the first line of Table 2.

The corresponding results for the lag 28 data are that 50.3% of days after January 28 had lower levels of exposure than their corresponding control days, with an average difference of 0.26 $\mu g/m^3$. The tendency for control days to have greater exposure is more pronounced than in the lag 14 data, and the effect of exposure is more severely underestimated.

Table 2: Comparison of Standard Case-crossover and Bidirectional Case-crossover Estimators

Estimator	True Value	Mean	SD	Nominal SE
$\hat{\beta}_{lag14}$	0.1000	0.0625	0.1707	0.1730
$\hat{\beta}_{lag28}$	0.1000	-0.0220	0.1708	0.1701
$\hat{\beta}_{1-1}$	0.1000	0.0993	0.1330	0.1412
$\hat{\beta}_{TH}$	0.1000	0.4894	0.0710	0.0856
$\hat{\beta}_{BI}$	0.1000	0.0995	0.0934	0.0988

The true value of β is 0.1. $\hat{\beta}_{lag14}$ and $\hat{\beta}_{lag28}$ are conventional case-crossover estimates, each using a single control day for each subject 14 or 28 days before failure, respectively. $\hat{\beta}_{1-1}$ is the bidirectional case-crossover estimate with a single control day for each subject randomly chosen from among the 363 control days. $\hat{\beta}_{TH}$ is a "total history" version of the case-crossover estimator, in which all days prior to failure are used as controls. $\hat{\beta}_{BI}$ is the bidirectional case-crossover estimator using the full stratum of 363 control days for each subject.

3.4 Application to USC Children's Health Study

During the calendar year 1994, a pilot school absence monitoring study was conducted at the University of Southern California. School absences for approximately 3600 Southern California children were recorded. The school absence monitoring study was part of the Children's Health Study, a large longitudinal study of the health effects of air pollution on children. We describe here an analysis in which the bidirectional case-crossover design was used to estimate acute effects of exposure to PM_{10} on the risk of absence from school.

The probability of absence for subject i on day j was modeled with the logistic model (1). The vector of covariates X_{ij} contained the peak PM_{10} level on the previous day, maximum temperature and humidity lagged one and two days, as well as indicators for the month and for the day of the week. This model ignores the fact that absences are autocorrelated, that is, if a subject is absent on a given day, the probability of absence the next day is greatly increased. In the analysis presented here, this was compensated for by treating only the first day of each period of absence as a failure, and discarding subsequent consecutive absences. Days on which the subject attended school were used as control days. Other approaches are possible. For example, one could include indicator functions for absence on the previous day, previous two days, etc., and interactions between these indicators and other covariates. In addition, since there is likely to be a waiting time of some duration between the end of one period of absence and the beginning of the next, one might include a function of the time since the last absence as a covariate.

Risks were estimated using the procedure described in section 3.3. The denominator in (6) was approximated by using a single set of control days as well as the case set. Results of

the analysis are presented in Tables 3 through 6.

Table 3 presents relative risks of absence in relation to peak ambient levels of O_3, PM_{10}, and NO_2 both one and two days prior to the start of the absence. No results are statistically significant, although peak O_3 lagged one day appears somewhat inversely related to absence risk.

Table 3: Relative Risk of Absence in Relation to Recent Exposure to Air Pollutants

Pollutant	Relative Risk per Unit Exposure	95 % C.I.
Peak O_3 lag 1	0.9778	0.9560–1.0001
Peak O_3 lag 2	1.0026	0.9813–1.0244
Peak PM_{10} lag 1	1.0082	0.9987–1.0018
Peak PM_{10} lag 2	0.9917	0.9819–1.0017
Peak NO_2 lag 1	0.9464	0.9273–1.0150
Peak NO_2 lag 2	1.0474	1.0242–1.0711

There were 2486 subjects for whom absences were reported. Adjusted for temperature and humidity lagged one and two days. Units are 10 ppb for O_3 and NO_2, 10 μg / m^3 for PM_{10}.

Table 4 presents relative risks of absence due to respiratory illness. No statistically significant results are observed. The sample size is much smaller than that for the analysis presented in Table 3, as is reflected in the increased width of the confidence intervals.

Table 4: Relative Risk of Absence Due to Respiratory Illness in Relation to Recent Exposure to Air Pollutants

Pollutant	Relative Risk per Unit Exposure	95 % C.I.
Peak O_3 lag 1	1.0295	0.9432–1.1238
Peak O_3 lag 2	0.9696	0.8970–1.0482
Peak PM_{10} lag 1	0.9832	0.9399–1.0285
Peak PM_{10} lag 2	0.9851	0.9488–1.0227
Peak NO_2 lag 1	1.0248	0.9457–1.1105
Peak NO_2 lag 2	0.9939	0.9076–1.0885

There were 499 subjects who reported an absence due to respiratory illness. Adjusted for temperature and humidity lagged one and two days. Units are 10 ppb for O_3 and NO_2, 10 μg / m^3 for PM_{10}.

Tables 5 and 6 present relative risks of absence due to respiratory illness for subjects with wheeze or with asthma, respectively. No statistically significant results are observed for wheezers, but peak NO_2 lagged two days is associated with a statistically significant

increase in risk for asthmatic subjects, and the risk associated with NO_2 lagged one day is elevated as well.

Table 5: Relative Risk of Absence Due to Respiratory Illness in Relation to Recent Exposure to Air Pollutants: Subjects With Wheeze Only

Pollutant	Relative Risk per Unit Exposure	95 % C.I.
Peak O_3 lag 1	0.9033	0.4552–1.7925
Peak O_3 lag 2	1.3696	0.7389–2.5385
Peak PM_{10} lag 1	0.8152	0.5275–1.2597
Peak PM_{10} lag 2	0.9210	0.5897–1.4384
Peak NO_2 lag 1	1.4720	0.9290–2.3324
Peak NO_2 lag 2	0.9117	0.5571–1.4921

There were 59 subjects with wheeze who reported an absence due to respiratory illness. Adjusted for temperature and humidity lagged one and two days. Units are 10 ppb for O_3 and NO_2, 10 μg / m^3 for PM_{10}.

Table 6: Relative Risk of Absence Due to Respiratory Illness in Relation to Recent Exposure to Air Pollutants: Subjects With Asthma Only

Pollutant	Relative Risk per Unit Exposure	95 % C.I.
Peak O_3 lag 1	0.7211	0.4904–1.0603
Peak O_3 lag 2	1.2892	0.8739–1.9018
Peak PM_{10} lag 1	0.9266	0.7933–1.0824
Peak PM_{10} lag 2	0.8251	0.6653–1.0232
Peak NO_2 lag 1	1.1096	0.8564–1.4377
Peak NO_2 lag 2	1.5034	1.0291–2.1962

There were 58 subjects with asthma who reported an absence due to respiratory illness. Adjusted for temperature and humidity lagged one and two days. Units are 10 ppb for O_3 and NO_2, 10 μg / m^3 for PM_{10}.

4 The Multilevel Analytic Design

4.1 Introduction

Epidemiologists recognize two basic strategies for looking at the association between an exposure and a disease: ecologic studies, in which disease rates in groups of individuals are related to the average exposure rates in these groups, and analytic studies, in which individuals' disease outcomes are related to their own exposure values. Cohort studies and case-control studies are examples of the latter type. The epidemiologic literature is full of examples of discrepancies between the conclusions of the two types of studies. In a classic example, Durkheim found suicide rates in provinces of Western Europe to be highly correlated with the proportion of Protestants. Regression analyses of these rates produced an estimate of the rate ratio for Protestants relative to Catholics of 7.5, compared with a value of 2 estimated on an individual basis (Selvin, 1958). Similarly, numerous associations between cancer rates and mean consumption of various dietary factors have been found in ecologic correlation studies, but establishing such associations at an individual level has proven more elusive (Prentice and Sheppard, 1990).

The resolution of such paradoxes usually turns on three issues: between-group confounding, measurement error, and restricted variability. Between-group confounding refers to a characteristic of groups that is not accounted for in the model but is the real risk factor. In the suicide example, such a factor might be the alienation felt by Catholics in predominantly Protestant provinces. This is the essential explanation of the "ecologic fallacy", in which spurious ecological associations may be caused by a tendency for the individuals in the higher exposure groups who get the disease not to have been exposed themselves but rather to have gotten the disease as a result of some other group characteristic. Exposure measurement error has different effects on the two types of studies, generally biasing associations at the individual level towards zero, but not at the aggregate level. Finally, studies conducted within a single group may have a restricted range of variation in exposure and hence limited power. Thus, in the diet example, the positive associations at the ecologic level might be explained by some confounding variable such as race that is not accounted for in the analysis, whereas the lack of association at the individual level might be due to dilution of a real effect by measurement error or by restricted variability in diet within racial groups.

Each of these designs has advantages and disadvantages. The main advantage of the ecologic design is cost, but the fact that there is often greater variation in exposure between groups than within groups is another advantage. On the other hand, ecologic studies typically suffer from between-group confounding (partly because groups will be more heterogeneous with respect to confounders than members of groups and partly because data on confounders are unavailable) and the exposure data are usually of poor quality (e.g., food disappearance rates rather than mean intake rates). Analytic studies are more readily controlled for confounding factors, and have better quality data, but may

suffer from the effects of measurement error and restricted variability.

Multilevel designs, in which both individual and aggregate level variables are used to explain individual outcomes, represent an attempt to capture the desirable features of both approaches. Von Korff et al., (1992) describe many ways in which multilevel analyses have been applied to problems in the social sciences, and recommend their application to epidemiology.

The design we will discuss here is a hybrid design that we shall call the "multilevel analytic design". Key to this design is an analysis that exploits both levels of comparison. Exposure and confounder data are assembled on individuals, to have the best possible quality. The resulting exposure-response relations can then be tested for compatibility with the between-group differences in rates, and if compatible, the two analyses can be pooled for greater power. In particular, this allows one to assess how much of the differences in disease rates between groups can be explained by differences in the distribution of risk factors.

We next provide some details about the basic design and its analysis. In the following section, we describe how the effects of measurement error may be incorporated. We then address the issue of design optimization, and provide an example with a simulation study. Finally, we describe an application to the USC Children's Health Study.

4.2 The Multilevel Analytic Design and Its Analysis

The design begins with a selection of a number of groups $g = 1, ..., G$, which might be defined by geographic areas (as in a study of air pollution), ethnicity (as in a study of diet), or any other factor for which group identifying data are readily available. Within each group, individuals $i = 1, ..., I_g$ are selected. (For notational simplicity, we set $I_g \equiv I$). Data on outcomes y_{gi}, exposures x_{gi}, and confounders v_{gi} are collected on each individual, and in addition, certain characteristics of the group X_g may also be collected. For example, in an air pollution study, individual exposure information might comprise personal exposure estimates (e.g., ozone badges), microenvironmental sampling (e.g., in homes, schools, cars, outdoors), or individual exposure modifying factors such as proportion of time spent outdoors or characteristics of their homes (air conditioning, presence of a smoker, heating and cooking sources, etc.). Group exposure characteristics might include estimates of the ambient levels from area monitoring. The specifics of the outcomes (continuous or binary, cross-sectional or longitudinal) and the sampling plan for individuals (survey, cohort, or case-control) will vary from study to study, but are not germane to the issues discussed here.

For conceptual and notational simplicity, we will assume that the outcome, exposure, and confounder are all univariate and continuous, and that the individuals in each group are chosen by simple random sampling. We also assume that the quantities of interest are

linearly related, that is,

$$y_{gi} = \alpha_g + \beta x_{gi} + \gamma v_{gi} + \varepsilon_{gi} \tag{12}$$

where α_g is the baseline outcome for group g and the ε_{gi} are independent random variables with $\mathrm{E}(\varepsilon_{gi}) = 0$, $\mathrm{Var}(\varepsilon_{gi}) = \sigma^2$. Interest centers on the estimation of β, the exposure effect.

The baseline effects α_g may be considered fixed or random. Considering them random may be appropriate when the groups on which data are collected are randomly chosen from a larger population of groups. If effects are fixed, then one parameter for each group is included in the model. If effects are random, then it is necessary only to include enough parameters to characterize their distribution. Usually this requires only two parameters, a mean and a variance. Therefore it is advantageous to treat effects as random when appropriate, so as to decrease the number of parameters in the model, resulting in more accurate estimation. The true exposures x_{gi} and the confounders v_{gi} may also be considered either fixed or random. Considering them random is appropriate, for example, when individual exposure is considered to be distributed around a function of ambient exposure and one or more exposure modifying variables, as will be described in section 4.4. In what follows we will consider α_g, x_{gi}, and v_{gi} to be random, and we make the following assumptions:

1. The random variables α_1, ..., α_G are independent and identically distributed (e.g., the groups are selected by simple random sampling).
2. The group baseline effects α_g are independent of both x_{gi} and v_{gi}.

In general, the true exposures x_{gi} will be unknown, and will be estimated by measured values, as discussed in the section 4.4. For the remainder of this section, in order to study the performance of our method under ideal circumstances, we will ignore the effect of measurement error, effectively assuming the true exposures to be known. We will also assume that the true values of the confounder v_{gi} are known, although measurement error in v_{gi} can bias the estimator of β (Greenland, 1980).

Eq. (12) can be used to estimate β, and is appropriate when the α_g's are considered fixed. When the α_g are independent random variables with $\mathrm{E}(\alpha_g) = \alpha$, $\mathrm{Var}(\alpha_g) = \tau^2$, an estimator with smaller variance is obtained using the equation

$$y_{gi} = \alpha + \beta x_{gi} + \gamma v_{gi} + \eta_{gi}. \tag{13}$$

The error η_{gi} is equal to $\alpha_g - \alpha + \varepsilon_{gi}$. The covariance matrix of $\boldsymbol{\eta}$ can be described as follows: Let $\rho = \tau^2/(\sigma^2 + \tau^2)$. Define $\boldsymbol{\Sigma} = (1-\rho)\mathbf{I} + \rho\mathbf{1}\mathbf{1}^T$, where $\mathbf{I}$ is the identity matrix and $\mathbf{1}$ is an I-dimensional column of 1s. Define $\boldsymbol{\Sigma}_{BIG}$ to be the $GI \times GI$ block diagonal matrix, consisting of G identical blocks of the matrix $\boldsymbol{\Sigma}$. Then the covariance matrix of $\boldsymbol{\eta}$ is equal to $(\sigma^2 + \tau^2)\boldsymbol{\Sigma}_{BIG}$. The matrix $\boldsymbol{\Sigma}^{-1}$ is equal to $a\mathbf{I} + b\mathbf{1}\mathbf{1}^T$, where $a = 1/(1-\rho)$, and $b = -\rho/\{[(I-1)\rho + 1](1-\rho)\}$. Thus $\boldsymbol{\Sigma}_{BIG}^{-1}$ is a block diagonal matrix with each block equal to $\boldsymbol{\Sigma}^{-1}$.

If ρ is known, the parameters α, β, and γ can be estimated by weighted least squares. If σ^2 and τ^2 are unknown, the parameters can be estimated by a two-stage procedure. In the first stage, only within groups differences are used. This is accomplished by using Eq. (12) to estimate the parameters $\alpha_1, ..., \alpha_G, \beta, \gamma$ by ordinary least squares. Denote by $\hat{\beta}_1$ the estimate of β obtained from this first-stage regression, and by $\hat{\sigma}^2$ the usual mean square residual estimate of error variance. The second stage regression involves only the between groups differences. The regression equation is obtained from Eq. (13) by averaging over i:

$$\bar{y}_{g.} = \alpha + \beta \bar{x}_{g.} + \gamma \bar{v}_{g.} + \bar{\eta}_{g.}. \tag{14}$$

The variables $\bar{\eta}_{g.}$ are independent with mean 0 and variance $\tau^2 + \sigma^2/I$. Denote by $\hat{\beta}_2$ the ordinary least squares estimate of β from Eq. (14). The mean square residual is an estimate of $\tau^2 + \sigma^2/I$, which can be combined with $\hat{\sigma}^2$ to yield an estimate of τ^2. The estimators $\hat{\beta}_1$ and $\hat{\beta}_2$ are uncorrelated. Let $\mathbf{X}_1$ and $\mathbf{X}_2$ denote the design matrices from the first and second stages, respectively. Then $\mathrm{Var}(\hat{\beta}_1)$ and $\mathrm{Var}(\hat{\beta}_2)$ are estimated with appropriate elements from the diagonals of the matrices $(\mathbf{X}_1^T\mathbf{X}_1)^{-1}\hat{\sigma}^2$ and $(\mathbf{X}_2^T\mathbf{X}_2)^{-1}(\hat{\sigma}^2/I + \hat{\tau}^2)$. The two-stage procedure is completed by computing the variance weighted average of $\hat{\beta}_1$ and $\hat{\beta}_2$ to obtain the estimator

$$\hat{\beta}_{pooled} = \frac{\mathrm{Var}(\hat{\beta}_2)\hat{\beta}_1 + \mathrm{Var}(\hat{\beta}_1)\hat{\beta}_2}{\mathrm{Var}(\hat{\beta}_1) + \mathrm{Var}(\hat{\beta}_2)}. \tag{15}$$

The relationship between weighted least squares and the two-stage procedure is given by the following:

Theorem: Let $\hat{\sigma}^2$, $\hat{\tau}^2$ be the estimators of σ^2, τ^2 from the two-stage procedure. Then $\hat{\beta}_{pooled}$ is the weighted least squares estimate of β when $\rho = \hat{\tau}^2/(\hat{\sigma}^2 + \hat{\tau}^2)$.

Corollary: If the errors η_{gi} are normally distributed, then the MLE of β satisfies Eq. (15), with $\hat{\beta}_{MLE}$ substituted for $\hat{\beta}_{pooled}$ and $\mathrm{Var}(\hat{\beta}_1)$ and $\mathrm{Var}(\hat{\beta}_2)$ evaluated at $\hat{\beta}_{MLE}$.

Proofs of these claims are provided in section 4.7. The corollary suggests that Eq. (15), if iterated, will converge to the MLE.

4.3 Potential Sources of Bias

As with any model, departures from model assumptions can lead to bias. This is especially true with ecologic analyses, for which many different types of bias have been documented. In the case where exposure is binary, Brenner et al., (1992) discuss bias that can result from nondifferential exposure misclassification in ecologic studies. This type of bias can occur in both linear and log-linear models, and is always away from the null.

Richardson et al., (1987) discuss biases resulting when the outcome is related to a non-linear function of exposure. They also discuss bias that results when baseline risks

within groups are related to mean exposures within groups. Greenland and Robins (1994) discuss bias resulting from nonlinearity and nonadditivity of effects. In particular, they show how interactions on the individual level may not be appropriately represented by the corresponding ecologic level interaction. That is, if the individual outcome y is related to individual level variables x_1 and x_2 by the relation

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_1 x_2, \tag{16}$$

and if μ_y, μ_{x_1}, μ_{x_2} are the mean levels in a given group, then the ecologic model

$$\mu_y = \beta_0 + \beta_1 \mu_{x_1} + \beta_2 \mu_{x_2} + \beta_3 \mu_{x_1} \mu_{x_2} \tag{17}$$

may give biased results.

While our model, like others, is subject to bias when its assumptions are violated, it should be relatively free from certain common ecologic biases due to the fact that exposure variables and confounders are assessed at the individual level. Therefore in the example given in equations (16) and (17), if we define μ_{12} to be the mean level of the interaction term $x_1 x_2$ in a particular group, our ecologic model would be

$$\mu_y = \beta_0 + \beta_1 \mu_{x_1} + \beta_2 \mu_{x_2} + \beta_3 \mu_{12}, \tag{18}$$

which does not suffer from nonadditivity bias.

With regard to bias resulting from a relationship between baseline risk and mean exposure across groups, this will not occur if our assumption of random selection of groups is met. If this assumption is violated, however, ecologic bias may result.

4.4 Allowance for Exposure Measurement Error

In many circumstances, it may not be feasible to obtain complete and error-free data on all individuals and hence some variables will only be available for some (randomly selected) subset of individuals. For example, in a dietary study, one might wish to validate the use of a food frequency questionnaire in the entire group by repeated 7-day records. In an air pollution study, it might be feasible to obtain personal monitoring or microenvironmental sampling data on only a sample, but questionnaire data on individual modifying factors might be available on the entire group. Optimization of the design would typically entail trade-offs between the number of groups and the number of individuals in the main study and in validation substudies, and the extent of the measurement protocols, subject to constraints on the total costs. These design issues will be discussed further below. In this section, we will focus on the effect of exposure measurement error. To simplify matters, we will ignore confounding. The extension of our results to the situation in which imperfectly

measured confounders are included is mathematically straightforward, but considerably more complex notationally.

We make a distinction in our analysis between two types of measurement error. The first type, known as the "Berkson" error model (Armstrong, 1990), applies when individuals are assigned their group average exposures. The second type, known as the "classical" error model, applies when the assigned exposure is a random variable whose expected value is the true exposure.

Let x_{gi} denote the unobservable true exposure for individual i in group g and let z_{gi} indicate the measured value (e.g., from personal monitoring). The classical error model assumes that the measured values are randomly distributed around the true value with the property that $\mathrm{E}(z_{gi}|x_{gi}) = x_{gi}$. As is well known (see Thomas, Stram, and Dwyer (1993) for a review), the classical error model produces a bias towards the null, essentially because the measured exposures are overdispersed ($\mathrm{Var}(z_{gi}) = \mathrm{Var}(x_{gi}) + \mathrm{Var}(z_{gi}|x_{gi}) > \mathrm{Var}(x_{gi})$). Thus if $\mathrm{Var}(x_{gi}) = \phi_g^2$ and $\mathrm{Var}(z_{gi}|x_{gi}) = \omega^2$, the regression on z_{gi} produces a slope estimate $\hat{\beta}$ that has expectation $c_g = \phi_g^2/(\phi_g^2 + \omega^2)$ times the expectation of the slope of the regression on the x_{gi}. This suggests a simple correction for measurement error if these variances are equal and known. First fit the naive regression on z_{gi} and then correct the estimated slope coefficient by dividing it by c_g (Rosner, Willett, and Spiegelman, 1989). For more complex situations, for example if the variances differ between groups, a useful strategy is to replace the z_{gi}'s by $\hat{x}_{gi} = \mathrm{E}(x_{gi}|z_{gi}) = c_g z_{gi} + (1 - c_g)\mathrm{E}(x_{gi})$ and then use these $\hat{x}_{gi}$'s as if they were the true exposures in the regression.

The Berkson error model assumes instead that the true exposures x_{gi} of individuals are distributed around their group estimates X_g with the property that $\mathrm{E}(x_{gi}|X_g) = X_g$. Thus, in an air pollution study with no personal monitoring, we might assume that individuals' exposures are randomly distributed around the ambient levels for their communities. A consequence of this assumption is that, at least for linear exposure-response models, the regression on the measured values provides unbiased estimates of the true slope. If $y_{gi} = \alpha_g + \beta x_{gi} + \varepsilon$, then

$$\begin{aligned} \mathrm{E}(y_{gi}|X_g) &= \alpha_g + \beta\mathrm{E}(x_{gi}|X_g) + \mathrm{E}(\varepsilon|X_g) \\ &= \alpha_g + \beta X_g. \end{aligned}$$

Thus, Berkson error produces no bias towards the null for linear models.

Typically, it would not be feasible to obtain true exposure data on any individuals. Rather, a surrogate variable w would be obtained on everybody and higher quality measurements z only on a sample. The measurements are assumed to be unbiased in the classical error sense and might be replicated T times. In this case, it will not be possible to use the z's directly in modeling y because they are available on too few subjects, but they could be used to build a model for the relationship between z and w which could then be used for imputing $\hat{x}$-values in the first stage regression. The surrogate variable w might be a simpler measure of x (such as a food frequency questionnaire) or it might be a personal modifier of

a group exposure characteristic X (for example, percent time spend outdoors in an air pollution study could modify the ambient pollution level).

To give a concrete example of this imputation procedure, assume that at times $t = 1, 2, ..., T$ we have measurements of a group exposure characteristic X_{gt} for each group, and for a subset of individuals we have an exposure modifying variable w_{git} and an exposure measurement z_{git}. We assume that X and w are assessed without error, and z has a classical error structure in relation to true exposure x. For the sake of simplicity, we will consider that the replicate measurements have been averaged over time, and we will drop the subscript t. We assume the following relationships:

$$x_{gi} \sim N(X_g + \delta_0 + \delta_1 w_{gi}, \phi^2) \tag{19}$$
$$z_{gi} \sim N(x_{gi}, \omega^2) \tag{20}$$

We assume that ω^2 is known from other studies or from another set of replicate measurements, but that δ_0, δ_1, and ϕ^2 are unknown. Combining Eqs. (19) and (20) yields

$$z_{gi} \sim N(X_g + \delta_0 + \delta_1 w_{gi}, \phi^2 + \omega^2) \tag{21}$$

from which we can obtain unbiased estimates of δ_0, δ_1, and ϕ^2 (since ω^2 is known). We then estimate x_{gi} as $\hat{x}_{gi} = X_g + \hat{\delta}_0 + \hat{\delta}_1 w_{gi}$, which is an unbiased estimator of $\mathrm{E}(x_{gi}|X_g, w_{gi})$ since $\hat{\delta}_0$ and $\hat{\delta}_1$ are unbiased.

Allowing for measurement error complicates the two-stage procedure for estimating the parameter β as follows. We assume

$$\alpha_g \sim N(\alpha, \tau^2) \tag{22}$$
$$y_{gi} \sim N(\alpha_g + \beta x_{gi}, \sigma^2) \tag{23}$$

where α, τ^2, and σ^2 are unknown. Since the x_{gi} are also unknown, we replace them with their estimates $\hat{x}_{gi}$ when fitting the model. The first stage model is thus:

$$y_{gi} = \alpha_g + \beta \hat{x}_{gi} + \varepsilon_{gi}, \tag{24}$$

where the ε_{gi} are independent normal random variables with $\mathrm{E}(\varepsilon_{gi}) = 0$ and $\mathrm{Var}(\varepsilon_{gi}) = \sigma^2 + \beta^2 \mathrm{E}[(x_{gi} - \hat{x}_{gi})^2 | X_g, w_{gi}]$.

Since $\mathrm{Var}(\varepsilon_{gi})$ depends on g, i, and on the unknown parameter β, the model Eq. (24) may be fit by iteratively reweighted least squares (IRLS). In order to use this procedure, we must first express the weights $\mathrm{Var}(\varepsilon_{gi})$ in usable form. Let $\hat{V}(\hat{\delta}_0)$, $\hat{V}(\hat{\delta}_1)$, and $\hat{C}(\hat{\delta}_0, \hat{\delta}_1)$, be the estimates of $\mathrm{Var}(\hat{\delta}_0 | X_g, \{w_{gi}\})$, $\mathrm{Var}(\hat{\delta}_1 | X_g, \{w_{gi}\})$, and $\mathrm{Cov}(\hat{\delta}_0, \hat{\delta}_1 | X_g, \{w_{gi}\})$, respectively, calculated from regression model Eq. (21). Since $\hat{x}_{gi} \approx \mathrm{E}(x_{gi}|X_g, w_{gi})$, $\mathrm{Var}(\varepsilon_{gi})$ can be approximated by $V^* = \sigma^2 + \beta^2 \mathrm{Var}(x_{gi}|X_g, w_{gi}) \approx \sigma^2 + \beta^2 W_{gi}$ where $W_{gi} = \hat{V}(\hat{\delta}_0) + 2w_{gi}\hat{C}(\hat{\delta}_0, \hat{\delta}_1) + w_{gi}^2 \hat{V}(\hat{\delta}_1) + \hat{\phi}^2$. The IRLS procedure is then conducted as follows. Set $\hat{\sigma}^2$ and $\hat{\beta}$ to arbitrary initial values, then fit model Eq. (24) by weighted least

squares regression using weights V^{*-1}. This produces an updated estimate $\hat{\beta}^{(1)}$ of β, and fitted values $\hat{y}_{gi}^{(1)}$. To obtain an updated estimate of σ^2, take the average of the values $(y_{gi} - \hat{y}_{gi}^{(1)})^2 - (\hat{\beta}^{(1)})^2 W_{gi}$ and then repeat the entire process.

Let $\hat{\alpha}_g$, $\hat{\beta}_1$, and $\hat{\sigma}^2$ be the estimates of α_g, β, and σ^2 obtained from the first-stage model Eq. (24). Let $\mathbf{W}$ be the diagonal matrix whose diagonal elements are $\hat{\sigma}^2 + \hat{\beta}_1 W_{gi}$. Let $\mathbf{X}$ be the design matrix corresponding to the first-stage model Eq. (24). The usual estimate of the covariance matrix of $\hat{\alpha}_g$, $\hat{\beta}_1$ is $(\mathbf{X}^T\mathbf{W}^{-1}\mathbf{X})^{-1}$, which is accurate when the substudy sample is reasonably large (see simulation results below).

The second-stage model is obtained from Eq. (24) by averaging over i and by replacing α_g with its mean α to obtain

$$\bar{y}_{g.} = \alpha + \beta \hat{x}_{g.} + \eta_g \tag{25}$$

where $\hat{x}_{g.} = I^{-1} \sum_{i=1}^{I} \hat{x}_{gi}$, and η_g are independent random variables with $\mathrm{E}(\eta_g) = 0$ and

$$\mathrm{Var}(\eta_g) = \tau^2 + \sigma^2/I + \beta^2 \mathrm{E}[(\bar{x}_{g.} - \hat{x}_{g.})^2 | X_g, \{w_{gi}\}]. \tag{26}$$

The expectation on the right hand side of Eq. (26) may be estimated with $\bar{W}_{g.}$, the average of the W_{gi} described above.

The second-stage model may also be fitted by IRLS, updating β as in the first-stage regression, and updating the sum $\tau^2 + \sigma^2/I$ as a single quantity. After convergence, a separate estimate of τ^2 can be obtained by combining the estimate of $\tau^2 + \sigma^2/I$ with the estimate of σ^2 from the first-stage regression. Let $\hat{\beta}_2$ be the estimate of β from the second-stage regression. The covariance matrix of $\hat{\alpha}$ and $\hat{\beta}_2$ can be obtained in a manner analogous to the method described above for the first-stage model.

If the true weights were known and did not depend on β, the estimates $\hat{\beta}_1$ and $\hat{\beta}_2$ would be uncorrelated. When variables are normally distributed, they are nearly uncorrelated when the weights are estimated as well (simulation results, not reported). For many applications in environmental epidemiology, it is more appropriate to assume that true exposures are nonnegative and lognormally distributed and that measurement errors are lognormally distributed and multiplicative. Furthermore, the individual exposure modifiers w_{gi} might also be assumed to act multiplicatively on the group means X_g. All this can be accomplished without any new theory simply by redefining x, X, and z to be the logarithms of their respective quantities.

4.5 Design Optimization

At the design stage, the epidemiologist needs to consider the trade-off between the number of groups and the number of subjects per group, the selection of the specific groups to be included, the number of subjects in the main study versus the number in the validation

sample, and the number and complexity of measurements to be made on each sample. These are important issues that have been given only limited attention in the context of analytic studies and none in the context of ecologic or hybrid designs. For analytic studies, Greenland (1988) and Spiegelman and Gray (1991) have considered the trade-offs between numbers of subjects in the main and validation studies and have provided explicit formulae for determining the optimal design where it is planned to use measurement error adjustment methods in the analysis like those described above. Rosner and Willett (1988) considered the trade-off between numbers of subjects and numbers of replicate measurements in a validation study.

For linear models with a continuous normally distributed outcome, ignoring confounding at the individual and group levels, measurement error, and assuming exposure is assessed only at the group level, the power of the study can be computed as a function of four quantities: the number of groups G, the number of subjects I sampled in each group, the true R^2 between group mean exposure and group mean outcome, and the ratio $VR = V_W/V_B$, where V_W and V_B are outcome variances within and between groups, respectively. Given these quantities, we compute $R_*^2 = IV_B R^2/(V_W + IV_B)$, the squared correlation between group mean exposure and the average outcome among the individuals sampled from the group. The quantity R_*^2 is less than R^2, because the sample mean outcome rather than the true group mean outcome is used. The power to detect a nonzero R^2 is calculated by using Fisher's transformation of R_*^2.

Table 7 illustrates the results for a variety of choices of the model and design parameters. It is clear that the power is much more strongly influenced by the number of groups than by the number of subjects. For a logistic model for binary outcomes, the power also depends on the overall disease frequency, but the same basic result emerges — the power of the aggregate analysis depends much more strongly on the number of groups than on the number of subjects per group.

Table 8 provides similar power calculations for testing a partial R^2 for the individual regression after removing group effects, again using Fisher's transformation. The power of these analyses depends only on the total number of individuals and it is clear that with sample sizes in the thousands, there is adequate power for detecting very small correlations. However, it is important to note that these are the correlations with the measured exposures, which could be severely attenuated by measurement error.

To provide further guidance for the design of the USC Children's Health study, we undertook a limited simulation study. For this purpose, we varied the number of groups G, the number of subjects per group in the main study I, and the number of subjects per group in the exposure substudy S. Relationships amongst the variables were as given in Eqs. (19–23), with the ambient levels X_g, X_{gt} and individual modifiers w_{gi}, w_{git} being normally distributed. For each choice of design parameters, 1000 replicate data sets were simulated and analyzed using the methods described above. We tabulated the bias and variance of the parameter estimates from the individual level regressions (with and without adjustment for measurement error), the ecologic regression, and the proposed pooled

Table 7: Statistical Power of Between-groups Comparisons

		R^2 for Between-groups Regression					
		$R^2 = 0.1$		$R^2 = 0.3$		$R^2 = 0.5$	
G	$G \times I$	$VR = 10$	$VR = 100$	$VR = 10$	$VR = 100$	$VR = 10$	$VR = 100$
5	1000	0.12	0.10	0.21	0.18	0.33	0.28
	4000	0.12	0.11	0.22	0.20	0.34	0.32
10	1000	0.21	0.15	0.46	0.32	0.72	0.54
	4000	0.21	0.19	0.48	0.43	0.75	0.68
20	1000	0.34	0.20	0.75	0.45	0.96	0.73
	4000	0.37	0.29	0.80	0.68	0.97	0.92
40	1000	0.52	0.23	0.94	0.54	1.00	0.84
	4000	0.60	0.41	0.97	0.86	1.00	0.99

G is the number of groups, and I is the number of individuals per group. $VR = V_W/V_B$, where V_W is the within-groups outcome variance and V_B is the between-groups outcome variance.

combination of the two regressions.

The design parameters were chosen to approximate those being considered for the USC Children's Health study, and the model parameters were then adjusted to illustrate a hypothetical situation in which the two approaches to estimation would be roughly equally informative. The true parameter values for the simulation are given in a footnote to Table 9. Table 9 illustrates the effect of modifying the design parameters under the constraint that the total number of measurements $G(I + SM)$ be fixed at 3000. Under the assumptions of the simulation, measurement error is minimized when one measurement is taken per individual in the substudy. Therefore we set $T = 1$. All parameter estimates appear to be nearly unbiased. The columns labeled "sample SD" give the sample standard

Table 8: Statistical Power of Within Groups Comparisons

$G \times I$	$R^2 = 0.001$	$R^2 = 0.005$	$R^2 = 0.01$	$R^2 = 0.05$
1000	0.26	0.72	0.94	1.00
2000	0.41	0.94	1.00	1.00
4000	0.64	0.99	1.00	1.00

Table 9: Standard Errors of Parameter Estimates in the Presence of Measurement Error

			$SE(\hat{\beta}_1)$		$SE(\hat{\beta}_2)$		$SE(\hat{\beta}_{pooled})$	
			Sample	Nominal	Sample	Nominal	Sample	Nominal
G	I	S	SE	SE	SE	SE	SE	SE
6	200	300	0.19	0.15	0.32	0.30	0.15	0.13
12	100	150	0.19	0.15	0.19	0.19	0.13	0.11
24	50	75	0.19	0.15	0.14	0.14	0.11	0.10
48	25	38	0.18	0.15	0.11	0.11	0.092	0.086
6	400	100	0.24	0.11	0.30	0.30	0.19	0.10
12	200	50	0.25	0.11	0.18	0.18	0.16	0.090
24	100	25	0.24	0.11	0.12	0.12	0.12	0.080
48	50	13	0.23	0.11	0.092	0.092	0.10	0.069

G is the number of groups, I is the number of individuals per group in the main study, and S is the number of individuals per group in the exposure validation substudy.
1000 data sets were generated for each value of G, I, and S.
Exposures measured subject to error; measurement error variance estimated in substudy.
True parameter values are $\phi^2 = 1.0$, $\omega^2 = 25.0$, $\tau^2 = 1.0$, $\sigma^2 = 25.0$. In addition, $\text{Var}(X_g) = 4.0$, $\text{Var}(X_{gt}|X_g) = 0.25$, $\text{Var}(w_{gi}) = 1.0$, and $\text{Var}(w_{git}|w_{gi}) = 0.25$.

deviation of the 1000 parameter estimates. The columns labeled "Nominal SE" give the square root of the average of the 1000 conditional variance estimates obtained from the covariance matrix estimator $(X^T W^{-1} X)^{-1}$. Each block of the table shows the effect of varying the number of groups and the number of subjects per group. In agreement with Tables 7 and 8, the efficiency of $\hat{\beta}_2$ improves rapidly as the number of groups increases, whereas $\hat{\beta}_1$ depends only on the total number of subjects. Comparing the two blocks illustrates the trade off between the number of subjects in the main study and validation substudy. The second-stage estimator is relatively insensitive to this parameter, while the first-stage estimator is improved by having a larger proportion in the validation study.

4.6 Application to the USC Children's Health Study

In January 1992, the California Air Resources Board (CARB) awarded a contract to the University of Southern California to initiate a 10-year cohort study of the health effects of air pollution in Southern California. The study enrolled a cohort of about 3500 school children from 12 communities selected so as to represent a variety of types and levels of air pollution that are represented in the basin. The primary focus of the study is on the effects of chronic exposure to one-hour peak ozone (O_3), but particulates (PM_{10}), nitrogen dioxide (NO_2), acids (H^+), and other pollutants are also being measured. Health outcomes

measured annually include various lung function tests, symptoms reported by questionnaire, and absences abstracted from school records.

4.6.1 Community Selection

Some preliminary power calculations based on assumed values for true effects indicated that for studying a single pollutant, it would be necessary to have at least 10 groups for power to be adequate. We carried out further calculations along similar lines to assess the prospects for doing multivariate analyses of two or more pollutants and concluded that it would be possible, provided groups could be selected in such a way that the correlations in pollutant levels across groups were not too large. Thus, the optimal choice would have to take account of the actual levels of exposure to each of the pollutants we wished to assess.

Fortunately, extensive data were available on the four highest priority pollutants from the CARB's monitoring program. Year-round average levels for the period 1986-1990 were obtained from 86 monitoring stations scattered across Southern California. (For some pollutants, notably acids, the values had to be interpolated from other stations on an inverse-distance weighted basis). Our initial selection of sites was based on the intuitive notions that (1) we wished to maximize the dispersion of each of the pollutants, and (2) we wished to represent as many combinations of high and low levels of each pollutant as possible.

For each pollutant, we calculated the mean level over the 86 communities, then for each community, we converted the pollution levels to standard units. Each community was assigned a "profile" by recording it as either above (+) or below (−) the mean level for each pollutant. For a design based on all four pollutants, there were thus $2^4 = 16$ possible profiles, of which demographically suitable examples could be found for 7 of them. Within each profile, we then selected from one to three communities whose sum of squared standardized pollution levels were large. Table 10 describes the characteristics of the communities that we judged to be the most suitable on this basis, under the constraint that we could afford to study no more than twelve. This selection process differs from the one described above in that the groups were not randomly chosen. Thus the group effects must be considered fixed rather than random.

In order to compare alternative designs based on different selections of priority pollutants, we then carried out a further simulation study, based purely on the second stage ecologic regression, but allowing the actual pollutant levels to differ from the measured values subject to a covariance structure estimated from the observed data. Table 11 summarizes the results of this simulation, which led us to the conclusion that, if all four pollutants had health effects, then the optimal design would need to be based on all four. This design appears to have adequate power for detecting differences in mean FEV_1 of about 3-5% between the high and low communities for each of the four pollutants in multivariate analysis, assuming that one-third of the variance in FEV_1 is explained by variation in the

Table 10: Characteristics of the Communities Selected for the University of Southern California Children's Health Study

Community	Profile	Annual Mean Level				Demographic Characteristics		
		O_3	PM_{10}	NO_2	H^+	% White	% Age 5-18	People/Room
Glendora	++++	109.2	67.0	39.1	2.93	89	20	0.50
Upland	++++	92.0	75.6	44.6	3.09	75	22	0.58
Mira Loma	+++−	95.1	84.9	32.8	1.05	68	24	0.62
Riverside	+++−	95.1	84.9	32.8	1.05	77	22	0.51
Perris	++−+	81.3	60.1	15.4	1.99	73	23	0.62
Lancaster	++−+	70.8	47.0	13.2	3.16	81	21	0.54
Lake Gregory	+−++	98.8	38.3	23.6	2.26	95	22	0.51
Alpine	+−−−	80.5	37.4	16.7	1.18	93	19	0.48
North Long Beach	−+++	45.2	49.5	44.8	2.43	58	18	0.59
Santa Maria	−−−−	30.2	28.0	7.7	0.91	66	20	0.61
Santa Barbara	−−−−	30.4	31.0	10.4	0.91	84	19	0.50
Lompoc	−−−−	34.8	30.0	1.6	0.91	72	21	0.58

In the "Profile" column, a "+" signifies that the pollution level is above the mean level of the 86 communities considered, a "−" signifies that the pollution is below that level. O_3 and NO_2 are measured in ppb on a mass basis. PM_{10} is measured in $\mu g/m^3$. H^+ is measured in ppb on a molar basis. The communities of Glendora, Perris, and Santa Barbara elected not to participate in the study, and were replaced by San Dimas, Lake Elsinore, and Atascadero, whose pollution characteristics were similar.

pollutants, and that O_3 and PM_{10} each contribute twice as much to the health effect as do NO_2 and H^+. Alternative designs that ignore one or more of these pollutants (with the same total number of communities) may slightly increase the variability of the pollutants of primary interest, which normally would be expected to yield an improvement in power. However, they also substantially weaken the power for controlling the confounding effect of the omitted pollutants and therefore in most instances reduce the power for the effects of interest in a multivariate analysis.

To determine whether we could significantly improve our selection of communities under the four-pollutant design, we conducted a final simulation along similar lines, starting with the choice given in Table 10 and in a stepwise fashion considered replacing each of the twelve communities by each of the remaining candidates. This led to the conclusion that, under an optimality criterion that maximized the sum of the powers for the four pollutants, it was theoretically possible to improve the design further by changing 5 of the 12 sites. This alternative choice attained better overall power by substantially reducing the correlations among the exposure variables. However, it did so at the expense of

Table 11: Comparison of Power to Detect Effects of Four Priority Pollutants from Alternative Choices of Sites

Community Selection Based On	Pollutants Included in Model	G	Power			
			O_3	PM_{10}	NO_2	H^+
O_3 PM_{10} H^+	O_3 PM_{10} NO_2 H^+	12	0.88	0.66	0.23	0.28
O_3 PM_{10} NO_2	O_3 PM_{10} NO_2 H^+	12	0.89	0.89	0.40	0.30
O_3 PM_{10} NO_2 H^+	O_3 PM_{10} NO_2 H^+	12	0.78	0.87	0.58	0.77
Random	O_3 PM_{10} NO_2 H^+	12	0.34	0.61	0.34	0.37
O_3 PM_{10}	O_3 PM_{10} NO_2 H^+	8	0.48	0.33	0.14	0.14
O_3 PM_{10} NO_2	O_3 PM_{10} NO_2 H^+	6	0.07	0.08	0.06	0.06
O_3 PM_{10} H^+	O_3 PM_{10} NO_2 H^+	6	0.13	0.10	0.07	0.07
Original	O_3 PM_{10}	5	0.22	0.23		

G is the number of groups in the study.

substantially reducing the variance of each exposure. Since we were unsure of the validity of the correlation estimates because many of the entries were based on interpolation, and since the overall improvement in power was modest, we decided to retain our original selection. Essentially, we judged that the primary objective of the study was to maximize the overall power to detect any air pollution effect and that the separation of the effects of particular pollutants was of only secondary importance, after having demonstrated an overall effect. We therefore felt that it was more important to maximize the variance in exposures than to minimize their covariances.

4.6.2 Exposure Modeling

The measurement protocol entails a combination of ambient monitoring, personal monitoring, microenvironmental sampling, and questionnaire assessment of personal modifying factors. Ambient data is routinely collected by the CARB for each of the communities, and provides long-term average levels throughout the study as well as historically. The questionnaire is administered to all subjects annually and includes items on residence history, usual indoor and outdoor times and activities and household characteristics (smoking by family members, air conditioning and heating, air exchange, sources of indoor pollution, etc.). Protocols for personal monitoring and microenvironmental sampling are still under discussion, and for this reason, we have not yet had the opportunity to bring the full force of our methods to bear on this study. We

describe below our analysis plan.

The goal of the analysis will be to combine these various data sources in such a way as to provide estimates of individual and group mean exposures for the first- and second-stage regressions described above, including estimates of measurement error distributions for adjustment purposes. The actual form of the models to be used is still under development, since it depends on the protocols adopted for the determination of personal exposure. To illustrate the general approach, we make some simplifying assumptions that will be remedied in our final analyses.

First, we assume that the relevant exposure variable is the long-term arithmetic mean (i.e, the time-weighted average, TWA). We also assume that ambient levels, true personal exposures, and measurement errors are lognormally distributed. Finally, we assume that the ratio of personal exposures to ambient levels is described by a multiplicative factor that depends loglinearly on the personal modifying factors. The basic relationships are thus as described in Eqs. (19)–(24), except for the additional complexities introduced by the lognormal assumptions. Using the estimates from this model, we can compute for each subject in the main study the TWA, $\mathrm{E}(e^{x_{gi}}|X_g, w_{gi})$, for use in the first-stage regression, together with the average over all subjects of these TWAs for use in the second-stage regression. Whether it will be possible to assess exposure effects at an individual level in a longitudinal analysis will depend primarily on the ability of the exposure model to accurately predict personal exposures. We have been successful in assessing exposure effects in the cross-sectional phase of the study, using lifetime exposures inferred from historical ambient measurements and residential histories. Even if it is not possible to assess exposure-response relations at an individual level, however, the use of average TWAs rather than X_g in the second stage should lead to more reliable estimates, because communities with different exposure patterns are likely to differ substantially in modifying factors such as use of air conditioning and proportion of time spent outdoors, because of major differences in climate across Southern California.

4.6.3 Estimation of Health Effects

We present an illustration of the multilevel procedure using the pulmonary function data in the USC Children's Health Study. More than 3500 children from 12 Southern California communities underwent spirometric assessment of pulmonary function. We determine the relationship between the measured FEV_1 and lifetime exposure to O_3. Lifetime exposure was assessed by compiling a residential history on each subject, then consulting historical records to determine the ambient levels of pollutants during each month of the subject's life.

The model used to analyze the data adjusted for exposure to the pollutants PM_{10} and NO_2, and for age, sex, race, height, and weight. The results were $\hat{\beta}_1 = -1.87 \pm 0.91$, $\hat{\beta}_2 = -0.16 \pm 0.97$, and $\hat{\beta}_{pooled} = -1.07 \pm 0.66$.

We were fortunate in the USC Children's Health study that, due to considerable levels of migration in Southern California, there was a large degree of variation within each community in the lifetime exposure to the pollutants under study. Therefore the individual level analysis provided a good level of efficiency for studying the effects of lifetime exposure, so we did not combine it with ecologic analysis in the Phase II report of this study (Peters, 1996). As described above, we expect the multilevel analytic design to play a considerable role in the longitudinal phase of the study.

4.7 Proofs

Proof of Theorem: We prove the theorem for a more general case with an arbitrary number of confounders. The model is

$$y_{gi} = \alpha + \beta x_{gi} + \mathbf{V}\gamma + \eta_{gi}$$

where $\mathbf{V}$ is a matrix of confounder variables. Let $\hat{\sigma}^2$ and $\hat{\tau}^2$ be estimates of σ^2 and τ^2, and let $\hat{\mathbf{\Sigma}}_{BIG}$ be the corresponding estimate of $\mathbf{\Sigma}_{BIG}$.

Assume without loss of generality that $\hat{\mathbf{\Sigma}}_{BIG}^{-1/2}\mathbf{x}$ is orthogonal to $\hat{\mathbf{\Sigma}}_{BIG}^{-1/2}\mathbf{V}$. Otherwise, replace $\mathbf{x}$ with $\mathbf{x} - \mathbf{V}(\mathbf{V}^T\hat{\mathbf{\Sigma}}_{BIG}^{-1}\mathbf{V})^{-1}\mathbf{V}^T\hat{\mathbf{\Sigma}}_{BIG}^{-1}\mathbf{x}$ and reparameterize the confounders γ. The weighted least squares estimates of α and β are the values minimizing

$$(\mathbf{y} - \alpha - \beta\mathbf{x})^T\hat{\mathbf{\Sigma}}_{BIG}^{-1}(\mathbf{y} - \alpha - \beta\mathbf{x}) \tag{27}$$

which is equal to

$$\sum_{g=1}^{G}(\mathbf{y}_g - \alpha - \beta\mathbf{x}_g)^T\hat{\mathbf{\Sigma}}^{-1}(\mathbf{y}_g - \alpha - \beta\mathbf{x}_g) \tag{28}$$

where $\mathbf{y}_g = (y_{g1}, ..., y_{gI})^T$, and $\mathbf{x}_g$ is defined similarly.

Let $\rho = \hat{\tau}^2/(\hat{\sigma}^2 + \hat{\tau}^2)$, $a = 1/(1-\rho)$, and $b = -\rho/(1-\rho)(1+(I-1)\rho)$. Substitute $(a\mathbf{I} + b\mathbf{1}\mathbf{1}^T)/(\hat{\sigma}^2 + \hat{\tau}^2)$ for $\hat{\mathbf{\Sigma}}^{-1}$ in Eq. (28) to obtain

$$\frac{1}{\hat{\sigma}^2 + \hat{\tau}^2}\sum_{g=1}^{G}\left[a\sum_{i=1}^{I}(y_{gi} - \alpha - \beta x_{gi})^2 + bI^2(\bar{y}_{g.} - \alpha - \beta\bar{x}_{g.})^2\right]. \tag{29}$$

Algebraic manipulation yields

$$\frac{a}{\hat{\sigma}^2 + \hat{\tau}^2}\sum_{g=1}^{G}\sum_{i=1}^{I}[y_{gi} - \bar{y}_{g.} - \beta(x_{gi} - \bar{x}_{g.})]^2 + \frac{Ia + I^2b}{\hat{\sigma}^2 + \hat{\tau}^2}\sum_{g=1}^{G}[\bar{y}_{g.} - \alpha - \beta\bar{x}_{g.}]^2. \tag{30}$$

Substitute appropriate expressions for a and b in terms of $\hat{\sigma}^2$ and $\hat{\tau}^2$ to obtain

$$\frac{1}{\hat{\sigma}^2}\sum_{g=1}^{G}\sum_{i=1}^{I}[y_{gi} - \bar{y}_{g.} - \beta(x_{gi} - \bar{x}_{g.})]^2 + \frac{1}{\hat{\sigma}^2/I + \hat{\tau}^2}\sum_{g=1}^{G}[\bar{y}_{g.} - \alpha - \beta\bar{x}_{g.}]^2. \tag{31}$$

Differentiating with respect to α and β and setting partial derivatives equal to 0 shows that the value of β minimizing Eq. (31) is

$$\hat{\beta}_{WLS} = \frac{\sum_g \sum_i (x_{gi} - \bar{x}_{g.})(y_{gi} - \bar{y}_{g.})/\hat{\sigma}^2 + \sum_g (\bar{x}_{g.} - \bar{x}_{..})(\bar{y}_{g.} - \bar{y}_{..})/(\hat{\sigma}^2/I + \hat{\tau}^2)}{\sum_g \sum_i (x_{gi} - \bar{x}_{g.})^2/\hat{\sigma}^2 + \sum_g (\bar{x}_{g.} - \bar{x}_{..})^2/(\hat{\sigma}^2/I + \hat{\tau}^2)}. \tag{32}$$

Let $\hat{V}_{\hat{\beta}_1} = \hat{\sigma}^2/\sum_g \sum_i (x_{gi} - \bar{x}_{g.})^2$ be the estimate of $\hat{\beta}_1$ and let $\hat{V}_{\hat{\beta}_2} = (\hat{\sigma}^2/I + \hat{\tau}^2)/\sum_g (\bar{x}_{g.} - \bar{x}_{..})^2$ be the estimate of $\hat{\beta}_2$ from the first- and second-stage models, respectively.

Multiplying numerator and denominator of Eq. (32) by $\hat{V}_{\hat{\beta}_1}\hat{V}_{\hat{\beta}_2}$ shows that

$$\hat{\beta}_{WLS} = \frac{\hat{V}_{\hat{\beta}_2}\hat{\beta}_1 + \hat{V}_{\hat{\beta}_1}\hat{\beta}_2}{\hat{V}_{\hat{\beta}_1} + \hat{V}_{\hat{\beta}_2}} = \hat{\beta}_{pooled}.$$

Proof of Corollary: If $\eta \sim N(0, \mathbf{\Sigma}_{BIG}^{-1})$, then the MLEs of α, β, σ, and τ are the values minimizing

$$L(\alpha, \beta, \sigma, \tau) = \log\left(\det(\mathbf{\Sigma}_{BIG})\right) + (\mathbf{y} - \alpha - \beta\mathbf{x})^T \mathbf{\Sigma}_{BIG}^{-1} (\mathbf{y} - \alpha - \beta\mathbf{x}). \tag{33}$$

Let $\nu^2 = \sigma^2/I + \tau^2$. The value of $\det(\mathbf{\Sigma}_{BIG})$ is $[(\sigma^2)^{I-1}\nu^2]^G$. Now

$$\begin{aligned} L(\alpha, \beta, \sigma, \tau) \;=\; L(\alpha, \beta, \sigma, \nu) \;&=\; G(I-1)\log(\sigma^2) + \tfrac{1}{\sigma^2}\textstyle\sum_{g=1}^{G}\sum_{i=1}^{I}[y_{gi} - \bar{y}_{g.} - \beta(x_{gi} - \bar{x}_{g.})]^2 \\ &+\; G\log(\sigma^2) + \tfrac{1}{\nu^2}\textstyle\sum_{g=1}^{G}[\bar{y}_{g.} - \alpha - \beta\bar{x}_{g.}]^2. \end{aligned} \tag{34}$$

For any given value of β, the values of α, β, σ, and ν minimizing L are

$$\begin{aligned} \hat{\alpha} &= \bar{y}_{..} - \beta\bar{x}_{..} \\ \hat{\sigma}^2 &= \frac{\sum_{g=1}^{G}\sum_{i=1}^{I}[y_{gi} - \bar{y}_{g.} - \beta(x_{gi} - \bar{x}_{g.})]^2}{G(I-1)} \\ \hat{\nu}^2 &= \frac{\sum_{g=1}^{G}[\bar{y}_{g.} - \bar{y}_{..} - \beta(\bar{x}_{g.} - \bar{x}_{..})]^2}{G}. \end{aligned}$$

For given values of σ^2, ν^2, it follows from the theorem that the value of β minimizing L is

$$\hat{\beta} = \frac{\sum_g \sum_i (x_{gi} - \bar{x}_{g.})(y_{gi} - \bar{y}_{g.})/\sigma^2 + \sum_g (\bar{x}_{g.} - \bar{x}_{..})(\bar{y}_{g.} - \bar{y}_{..})/\nu^2}{\sum_g \sum_i (x_{gi} - \bar{x}_{g.})^2/\sigma^2 + \sum_g (\bar{x}_{g.} - \bar{x}_{..})^2/\nu^2},$$

which is Eq. (15).

5 Measurement Error in Air Pollution Exposure Assessment

5.1 Introduction

In epidemiologic studies, cumulative exposure during a period of time is generally thought of as the integral of the function that expresses the concentration of the substance to which the subject is exposed in terms of time. This can be written

$$E = \int_0^T C(t)dt. \tag{35}$$

Exposure assessment methods attempt to estimate the integral on the right hand side of (35).

We considered the assessment of cumulative exposure to an air pollutant over a fixed time interval. We studied two classes of methods, the indirect or microenvironmental approach (Fugas, 1975; Duan, 1982), and the direct or personal sampling approach (e.g. Koutrakis et al., 1991, 1993). Using a simple model for true exposure, we compared the accuracy of exposure assessments under each method under a variety of assumptions about the magnitudes of the measurement errors involved. We also assessed the impact of these errors on a hypothetical model that describes the effect of exposure on a health outcome.

5.2 The Microenvironmental Method

In the microenvironmental method, the region occupied by an individual during the time period of interest is divided into a number of microenvironments, e.g. home, school, office, car, outdoor areas, etc. The cumulative exposure is expressed in terms of the concentrations of the pollutant of interest and the lengths of time spent in each microenvironment. Let $M_1, ..., M_n$ represent a complete list of the microenvironments occupied by an individual, let t_m be the length of time during which microenvironment M_m is occupied, and let C_m be the average concentration of the pollutant of interest in M_m during the time that M_m is occupied. Then

$$E = \sum_{m=1}^{n} C_m t_m. \tag{36}$$

Estimation of E involves specifying the microenvironments, then estimating the concentrations C_m and the times t_m. Estimates of the C_m are generally based on measured ambient levels in outdoor locations, indoor levels, and indoor emission strengths. Estimates of the t_m are generally based on questionnaires or diary information. There are several exposure models available to compute the estimates of C_m and t_m. They can be classified

as statistical models, physical models, and physical-stochastic models (Sexton and Ryan, 1988; Ryan, 1991). Statistical models are based on statistical analyses of actual exposures and factors thought to influence them. Physical and physical-stochastic models are based on physical laws. Examples include the SHAPE model (Ott, 1984; Ott et al., 1988), the NEM and pNEM models (Johnson and Paul, 1983; McCurdy and Johnson, 1989), the SIMSYS model (Ryan et al., 1986), the NEM-SAI (Hayes 1989), and the REHEX model (Winer et al., 1989; Lurmann et al., 1989, Lurmann and Colome 1991). Some models, such as pNEM and REHEX, also estimate the dose received by the subjects as the product of the concentration and the ventilation rate. Dose may be a more relevant metric than exposure for epidemiologic research and ultimately all exposure models may need to address this parameter. However, the current analysis is limited to exposure in order to reduce the complexity of the analysis and because errors in assignment of ventilation rates are likely to be large.

In most models, the microenvironmental concentration is expressed as the product of the ambient level in an outdoor location and a proportionality factor (e.g. indoor/outdoor ratio), plus a term representing the contribution from non-ambient sources in the microenvironment. Thus, the microenvironmental concentration can be expressed in the form

$$C_m = f_m A + S_m, \tag{37}$$

where A is the ambient level, f_m is an estimated constant of proportionality expressing microenvironmental concentration as a fraction of the ambient level, and S_m is the concentration from non-ambient sources.

Efforts to validate microenvironmentally based methods of exposure assessment have been quite limited. There is some evidence that these models tend to estimate mean population exposure well, but are rather inaccurate in describing the tails of the distribution (Ott et al., 1988). No physical model has been adequately evaluated for ozone (Strock et al., 1985; Johnson et al., 1990; Liu et al., 1993; Liu et al., 1995, McCurdy 1994).

5.3 The Personal Sampling Method

Integrated personal sampling devices (badges) are instruments worn by individuals that attempt to measure the exposure of the individual to a pollutant. Continuous instruments, which can provide 1-minute average concentrations for up to 48 hours of sampling, are available for personal sampling of some pollutants, such as carbon monoxide. Development of continuous ozone monitors has begun (Penrose et al., 1994; Topham et al., 1992). Most personal sampling devices, however, can only measure the cumulative exposure over the period that they are worn. Both passive and active (pumped) badges are manufactured. Passive badges consist of a diffusion barrier followed by an impregnated filter with which the depositing pollutant reacts. Recently developed passive ozone badges include the Koutrakis sampler (Koutrakis et al., 1991, 1993; Liu et al., 1994), the DGA ozone badge

(Grosjean and Hishani, 1992), and the Kanno-Yanagisawa ozone badge (Kanno et al., 1992). Passive badges are also used for sampling NO_2 (Palmes et al., 1976) and formaldehyde (Geisling et al., 1982). Most personal samplers for particulates are active, involving the pumping of air through a filter that traps the particles. The filter is weighed both before and after sampling to determine the concentration. Avol et al. (1989) evaluated a pumped ozone badge that uses a solid monitoring reagent. The reagent develops a visible color change that is quantifiable with acetone extraction and spectrophotometric analysis. They concluded that this sampler was appropriate only for exposure periods of a few hours or less. Geyh et al. (1994) have reported on an active ozone sampler that is suitable for sampling periods up to 48 hours and is more accurate and precise than the Koutrakis passive sampler.

Integrated personal sampling devices are subject to several types of measurement error. Probably most serious for passive badges is sensitivity to wind velocity, since wind blowing into the badge effectively reduces the length of the diffusion chamber, increasing the deposit rate. Readings can vary according to badge placement. Placement near the nose is probably most accurate, but not necessarily most practical. Finally interference with other pollutants can occur. Personal samplers are probably less accurate for ozone than for other pollutants, both because personal ozone sampling technology is new, and because ozone is more reactive than most other pollutants.

5.4 Modeling the Microenvironmental and Personal Sampler Methods

The microenvironmental and personal sampling methods differ with respect to two principles. First, in the microenvironmental method, concentrations are measured with a model chosen by the experimenter. Thus many different exposure metrics can be handled. The method may be used to estimate peak exposure or proportion of time above a certain concentration, as well as cumulative exposure. In contrast, integrated personal sampling devices can only measure cumulative exposure for most pollutants. The second difference, which is quite important, is that while the microenvironmental method requires specifications of microenvironments and estimation of time durations, both of which are subject to error, the personal sampler always spends the correct amount of time in each microenvironment. Thus in terms of equation (36), the personal sampler is subject to error only in the measurement of the C_m, while the microenvironmental method is subject to measurement errors in the t_m as well.

To make a more detailed comparison of the two procedures, we model the measurement error in each, as well as the true exposure process. In practice there may be other sources of error, such as non-response bias, that might affect the accuracy of these procedures. We do not consider these here. We model a study involving I hypothetical communities with J subjects in each community, and we assume that each subject spends time in K

microenvironments. Let A_i be the true ambient ozone level in community i. For each microenvironment $m = 1, 2, ..., K$, let f_{ijm} be the concentration in microenvironment m to which subject j in community i is exposed, expressed as a fraction of the ambient level A_i, and let t_{ijm} be the duration of time spent in microenvironment m by subject j in community i. We ignore the contribution from non-ambient sources. It follows that the true exposure of subject ij is given by

$$E_{ij} = \sum_{m=1}^{K} f_{ijm} t_{ijm} A_i. \tag{38}$$

We model the assessment processes of both the microenvironmental and the personal sampling methods. We assume that estimated values of f_{ijm} and t_{ijm} are normally distributed around the true values with a standard deviation proportional to the true value. Since these values are subject to physical constraints, e.g. indoor/outdoor ratios are generally required to lie between 0 and 1, we must assume that the standard deviations are small enough so that virtually all of the mass of the normal curve lies within these limits. This is the case for the standard deviation values we used in our simulations below. For the personal sampling method we specify a standard deviation ν, then generate an estimate $\hat{f}^P_{ijm} \sim N(f_{ijm}, \nu^2 f^2_{ijm})$ for each subject and microenvironment. The personal sampling estimate of exposure is then

$$\hat{E}^P_{ij} = \sum_{m=1}^{K} \hat{f}^P_{ijm} t_{ijm} A_i. \tag{39}$$

For the microenvironmental procedure, we specify a standard deviation τ_f, then generate an estimate $\hat{f}^M_{ijm} \sim N(f_{ijm}, \tau_f^2 f^2_{ijm})$ for each subject and microenvironment. To model the errors in estimating the t_{ijm}, we specify a standard deviation τ_t, then generate random errors $\varepsilon_{ijm} \sim N(0, \tau_t^2 t^2_{ijm})$ for each subject and microenvironment. We then set the estimate $\hat{t}^M_{ijm} = t_{ijm} + \varepsilon_{ijm} - \sum_{m=1}^{K} \varepsilon_{ijm}/K$. In this way $\sum_{m=1}^{K} \hat{t}^M_{ijm} = \sum_{m=1}^{K} t_{ijm}$ as required. The microenvironmental estimate of exposure is

$$\hat{E}^M_{ij} = \sum_{m=1}^{K} \hat{f}^M_{ijm} \hat{t}^M_{ijm} A_i. \tag{40}$$

Under our model, the estimates $\hat{f}^M_{ijm}$, $\hat{t}^M_{ijm}$, and $\hat{f}^P_{ijm}$ are unbiased, and for any given subject and microenvironment, the values $\hat{f}^M_{ijm}$ and $\hat{t}^M_{ijm}$ are independent. It follows that $\hat{E}^P_{ij}$ and $\hat{E}^M_{ij}$ are both unbiased for the true exposure E_{ij}, so the performances of the two estimators can be measured by comparing their variances. The variance of $\hat{E}^P_{ij}$ is

$$\mathrm{Var}(\hat{E}^P_{ij}) = \sum_{m=1}^{K} \nu^2 f^2_{ijm} t^2_{ijm} {A_i}^2. \tag{41}$$

The variance of $\hat{E}^M_{ij}$ is more complicated, since two random errors are involved, and since the errors in the $\hat{t}^M_{ijm}$ are correlated. Let $\mathbf{\Sigma}$ be the $K \times K$ covariance matrix of the $\hat{t}^M_{ijm}$. The kl element of $\mathbf{\Sigma}$ is

$$\mathrm{Cov}(t^{(M)}_{ijk}, t^{(M)}_{ijl}) = \begin{cases} \tau_t^2 \sum_{m=1}^{K} (t_{ijm}/K)^2 - t^2_{ijk}/K - t^2_{ijl}/K & \text{if } k \neq l \\ \tau_t^2 \sum_{m=1}^{K} (t_{ijm}/K)^2 + (K-2) t^2_{ijk}/K & \text{if } k = l. \end{cases} \tag{42}$$

Let $\mathbf{f_{ij}}$ be the vector $(f_{ij1}, ..., f_{ijK})$ considered as a column. The variance of $\hat{E}_{ij}^M$ is

$$\mathrm{Var}(\hat{E}_{ij}^M) = A_i{}^2(\mathbf{f_{ij}}{}^T \mathbf{\Sigma f_{ij}} + \tau_f^2 \sum_{m=1}^K f_{ijm}^2 t_{ijm}^2 + \tau_f^2 \tau_t^2 \sum_{m=1}^K f_{ijm}^2 [(K-2)t_{ijm}^2/K + \sum_{m=1}^K (t_{ijm}/K)^2]). \quad (43)$$

5.5 Estimating Health Effects — A Simulation Study

To assess the impact of measurement error on the estimation of health effects, we analyzed a linear model in which a health outcome (e.g. $\mathrm{FEV_1}$) is modeled as a linear function of exposure. We assume we have J subjects in each of I communities. The model is

$$F_{ij} = \alpha_i + \beta E_{ij} + \varepsilon_{ij}, \quad (44)$$

where F_{ij} is $\mathrm{FEV_1}$ (or other health outcome) measured on the jth subject in the ith community, expressed as a percentage of a baseline value for that subject. The baseline may be derived from other measurements of the same subject taken under relatively pollution-free conditions, or from measurements on a control group matched on variables such as height, weight, sex, race and age. The value α_i is a community-specific intercept, E_{ij} is the true exposure of subject ij, and ε_{ij} is a random error.

In order to perform the simulation, we specified values for the parameters in the model. We set the number of communities I to 12 and the number of subjects J per community to 300, to match the California Air Resources Board Children's Health Study. For the sake of simplicity, we assumed that each subject spends time in only three microenvironments: home, school, and outdoors. To compute "true" exposures E_{ij}, we specified values f_{ijm}, t_{ijm}, and A_i as follows: For the A_i we took the average daily maximum ozone concentrations during the years 1986–1990 for the 12 communities in the CARB Children's Health Study. These communities and the corresponding ambient levels are given in Table 12. To generate true values for the f_{ijm}, we specified a mean μ and a standard deviation σ for the ratios of the concentrations in each microenvironment to the ambient level A_i measured at a monitoring station. These specifications were based on the available literature on indoor/outdoor ozone ratios. Yocum (1982) concluded in a review that indoor/outdoor ratios generally fall between 0.1 and 0.7. Druzik et al. (1989) found ratios ranging from 0.24 to 0.75 for a variety of buildings. Wechsler (1989) studied three indoor sites with very different ventilation rates and found ratios ranging from 0.24 to 0.71. There appears to be little work available on residences as opposed to public buildings. To be consistent with published findings for buildings, we specified a mean ratio $\mu = 0.5$ and a standard deviation of $\sigma = 0.1$ for schools. For homes we took $\mu = 0.5$ and $\sigma = 0.15$, on the assumption that ratios would vary somewhat more from house to house than from school to school. Somewhat arbitrarily, we took $\mu = 1$ and $\sigma = 0.2$ for ratios of outdoor concentrations to the values A_i measured at monitoring stations. Then for each subject ij and each microenvironment m, we generated a true value f_{ijm} by generating an observation

from the normal distribution with mean and standard deviation appropriate to the microenvironment.

Table 12: Twelve Communities and Their Mean Peak Ozone Levels

Community	Ozone Level
Alpine	80.5
Lake Elsinore	82.7
Lake Gregory	98.9
Lancaster	70.8
Lompoc	42.7
Long Beach	45.2
Mira Loma	95.1
Riverside	95.1
San Dimas	109.2
Atascadero	58.7
Santa Maria	30.2
Upland	92.0

Measurements are average daily maximum for the period 1986–1990.
Units are ppb.

In specifying values for the times t_{ijm} spent in the various microenvironments, we were constrained by the requirement that $\sum_{m=1}^{K} t_{ijm} = 24$, when t_{ijm} is measured in hours per day. We therefore generated preliminary values t^*_{ijm} for each m and took $t_{ijm} = 24t^*_{ijm}/\sum_{m=1}^{K} t^*_{ijm}$. To generate the values t^*_{ijm} we used the normal distribution, specifying a mean μ and standard deviation σ for each microenvironment. To specify the mean value for percentage of time spent outdoors, we relied on findings of Wiley (1991) that indicated that California schoolchildren spend about 10% of their time outdoors on average. That percentage stays approximately constant over different times of the year, and on weekends as well as weekdays. We therefore took $\mu = 3$ and $\sigma = 1$ for the number of hours per day spent outdoors. We took $\mu = 6$ and $\sigma = 2$ for the number of hours in school, and $\mu = 15$ and $\sigma = 2$ for the number of hours spent at home.

Our simulation was based on 1000 artificial data sets. For each data set we first generated for each subject a "true" exposure E_{ij} computed from (38). We then generated measured exposures $\hat{E}^M_{ij}$ and $\hat{E}^P_{ij}$ as described in section 5.4, using one of several values for the standard deviations ν, τ_f, and τ_t. To fit model (44), we took ε_{ij} to be normally distributed with mean 0 and standard deviation 13. This standard deviation was estimated from between-individuals data on approximately 3600 Southern California school children tested in the spring of 1993 in the CARB Children's Health Study.

Our model assumes that measured values are normally distributed around the true values. Therefore it should provide reasonably accurate results whenever measured values are

distributed reasonably symmetrically around the true values. If measurements are severely biased, or skewed as a result of outliers, the results may be different that those described here.

We took the exposure-response slope β equal to -0.10, indicating a 1% decrease in FEV_1 per 10 ppb increase in exposure. All the α_i were taken to have the common value $100 - \beta\bar{E}$, where $\bar{E}$ is the average of the true exposures E_{ij} over all subjects in all communities. In this way the expected value of F_{ij} is 100, as required.

The variances of $\hat{E}_{ij}^M$ and $\hat{E}_{ij}^P$ were well estimated. Table 13 shows true and estimated variances of $\hat{E}_{ij}^M$ and $\hat{E}_{ij}^P$ averaged over i and j for several values of τ_f^2, τ_m^2, and ν^2. True and estimated variances were computed as described in section 5.4.

Table 13: True and Estimated Variances of Microenvironmental and Personal Sampler Estimates of Exposure

Exposure Estimate	True Variance	Estimated Variance
Microenvironmental ($\hat{E}_{ij}^M$)		
$\tau_f = .1$, $\tau_t = .1$	10.25	10.31
$\tau_f = .1$, $\tau_t = .2$	14.58	14.78
$\tau_f = .1$, $\tau_t = .3$	21.78	22.15
$\tau_f = .2$, $\tau_t = .1$	36.95	37.18
$\tau_f = .2$, $\tau_t = .2$	42.00	42.88
$\tau_f = .2$, $\tau_t = .3$	50.42	52.26
$\tau_f = .3$, $\tau_t = .1$	81.43	81.91
$\tau_f = .3$, $\tau_t = .2$	87.68	89.63
$\tau_f = .3$, $\tau_t = .3$	98.18	102.27
Personal Sampler ($\hat{E}_{ij}^P$)		
$\nu = .05$	2.20	2.20
$\nu = .10$	8.81	8.82
$\nu = .15$	19.84	19.84
$\nu = .20$	35.27	35.26
$\nu = .25$	55.11	55.10
$\nu = .30$	79.39	80.38

Variances are the averages over all subjects ij of $\text{Var}(\hat{E}_{ij}^M)$ and $\text{Var}(\hat{E}_{ij}^P)$.

We fit model (44) using $\hat{E}_{ij}^M$ and $\hat{E}_{ij}^P$ in turn in place of the unknown E_{ij}. We also fit an ecological version of the model in which all the α_i were assumed equal, and in which the ambient level A_i was used to estimate the true exposure E_{ij} for all the subjects in community i. Table 14 gives results based on 1000 simulated data sets. For each data set the least squares estimate $\hat{\beta}$ and the conventional estimate of its standard error was

Table 14: Performance of Dose-response Slope Estimator When FEV_1 is Regressed Against Various Estimates of Exposure: No Bias Correction Applied

Exposure Estimate	Mean	SD	Nominal SE	Bias	Root Mean Square Error
True Exposures	-0.099	0.027	0.027	0.001	0.027
Outdoor Ambient Level	-0.057	0.009	0.009	0.043	0.044
Microenvironmental ($\hat{E}_{ij}^M$)					
$\tau_f = .1$, $\tau_t = .1$	-0.086	0.025	0.025	0.014	0.029
$\tau_f = .1$, $\tau_t = .2$	-0.083	0.024	0.024	0.017	0.030
$\tau_f = .1$, $\tau_t = .3$	-0.076	0.024	0.023	0.024	0.034
$\tau_f = .2$, $\tau_t = .1$	-0.064	0.021	0.021	0.036	0.042
$\tau_f = .2$, $\tau_t = .2$	-0.061	0.020	0.021	0.039	0.044
$\tau_f = .2$, $\tau_t = .3$	-0.057	0.020	0.020	0.043	0.047
$\tau_f = .3$, $\tau_t = .1$	-0.045	0.018	0.018	0.055	0.058
$\tau_f = .3$, $\tau_t = .2$	-0.042	0.018	0.018	0.058	0.060
$\tau_f = .3$, $\tau_t = .3$	-0.040	0.017	0.017	0.060	0.063
Personal Sampler ($\hat{E}_{ij}^P$)					
$\nu = .05$	-0.097	0.026	0.026	0.003	0.026
$\nu = .10$	-0.087	0.025	0.025	0.013	0.028
$\nu = .15$	-0.077	0.023	0.023	0.022	0.032
$\nu = .20$	-0.065	0.022	0.022	0.035	0.041
$\nu = .25$	-0.053	0.021	0.020	0.046	0.051
$\nu = .30$	-0.045	0.019	0.018	0.055	0.058

True value of parameter is -0.10. Sample size is 3600.

calculated. The column labeled "Mean" is the average of the 1000 values of $\hat{\beta}$. The column labeled "SD" is the sample standard deviation of these 1000 estimates. This is a consistent estimate of the true standard error when the true exposure is estimated. The column labeled "Nominal SE" is the square root of the average of the 1000 conventional estimates of $\text{Var}(\hat{\beta})$. The column labeled "Bias" gives the difference between the mean and the true value of -0.10. The column labeled "Root Mean Square Error" gives the quantity $\sqrt{\text{SE}^2 + \text{Bias}^2}$, which is the usual measure of the accuracy of an estimator. The first row of the table gives the results when the true exposures E_{ij} are used as the dependent variable. The estimate is unbiased. In comparison, estimates based on the ambient level, $\hat{E}_{ij}^M$, or $\hat{E}_{ij}^P$, are biased severely toward 0. In the case of $\hat{E}_{ij}^M$ and $\hat{E}_{ij}^P$, the bias is due to nondifferential measurement error. In the case of the ambient level, it is due to the fact that the ambient level strongly overestimates the exposure, since it is equivalent to assuming that subjects are outside all the time. In principle, the conventional estimates of the standard deviation of $\hat{\beta}$ should tend to underestimate the true standard error slightly. This should be the case

Table 15: Performance of Exposure-response Slope Estimator When FEV_1 is Regressed Against Various Estimates of Exposure: Bias Correction Applied

Exposure Estimate	Mean	SD	Nominal SE	Bias	Root Mean Square Error
True Exposures	-0.099	0.027	0.027	0.001	0.027
Outdoor Ambient Level	-0.057*	0.009	0.009	0.043	0.044
Microenvironmental ($\hat{E}_{ij}^M$)					
$\tau_f = .1$, $\tau_t = .1$	-0.099	0.029	0.029	0.001	0.029
$\tau_f = .1$, $\tau_t = .2$	-0.101	0.029	0.030	-0.001	0.029
$\tau_f = .1$, $\tau_t = .3$	-0.101	0.032	0.031	-0.001	0.032
$\tau_f = .2$, $\tau_t = .1$	-0.100	0.034	0.034	-0.000	0.034
$\tau_f = .2$, $\tau_t = .2$	-0.101	0.033	0.034	-0.001	0.033
$\tau_f = .2$, $\tau_t = .3$	-0.102	0.035	0.036	-0.002	0.035
$\tau_f = .3$, $\tau_t = .1$	-0.102	0.040	0.040	-0.002	0.040
$\tau_f = .3$, $\tau_t = .2$	-0.100	0.042	0.041	0.000	0.042
$\tau_f = .3$, $\tau_t = .3$	-0.100	0.043	0.043	-0.000	0.043
Personal Sampler ($\hat{E}_{ij}^P$)					
$\nu = .05$	-0.100	0.029	0.030	-0.000	0.027
$\nu = .10$	-0.099	0.029	0.029	0.001	0.029
$\nu = .15$	-0.101	0.030	0.031	-0.001	0.030
$\nu = .20$	-0.100	0.034	0.033	-0.000	0.034
$\nu = .25$	-0.099	0.038	0.036	0.001	0.038
$\nu = .30$	-0.099	0.041	0.040	0.001	0.041

*No bias correction is applied to the ambient level estimate.

True value of parameter is -0.10. Sample size is 3600.

because the conventional estimator $\hat{\sigma}^2 / \sum_{i=1}^{I} \sum_{j=1}^{J} (\hat{E}_{ij} - \hat{E}_{i.})^2$ is a consistent estimator of the expectation of the conditional variance $\mathrm{Var}(\hat{\beta}|\hat{E}_{ij})$. The desired variance is the unconditional variance $\mathrm{Var}(\hat{\beta})$, which is equal to $\mathrm{E}[\mathrm{Var}(\hat{\beta}|\hat{E}_{ij})] + \mathrm{Var}[\mathrm{E}(\hat{\beta}|\hat{E}_{ij})]$, where $\mathrm{E}(\hat{\beta}|\hat{E}_{ij}) = \beta \sum_{i=1}^{I} \sum_{j=1}^{J} (\hat{E}_{ij} - \hat{E}_{i.})(E_{ij} - E_{i.}) / \sum_{i=1}^{I} \sum_{j=1}^{J} (\hat{E}_{ij} - \hat{E}_{i.})^2$. Since this quantity is not constant, its variance is strictly positive. However, the variance tends to 0 as the product $IJ \to \infty$, and appears to be negligible in our simulation.

If the parameters τ_f^2, τ_t^2, and ν^2 are known, the variances of $\hat{E}_{ij}^P$ and $\hat{E}_{ij}^M$ can be estimated, and the bias in $\hat{\beta}$ can be corrected. A consistent estimator of $\hat{\beta}$ is obtained by multiplying the conventional estimator by the correction factor

$$F = \frac{\sum_{i=1}^{I} \sum_{j=1}^{J} (\hat{E}_{ij} - \hat{E}_{i.})^2}{\sum_{i=1}^{I} \sum_{j=1}^{J} (\hat{E}_{ij} - \hat{E}_{i.})^2 - \frac{J}{J-1} \sum_{i=1}^{I} \sum_{j=1}^{J} \mathrm{Var}(\hat{E}_{ij})} \tag{45}$$

The denominator of the expression (45) is an unbiased and consistent estimator of $\sum_{i=1}^{I}\sum_{j=1}^{J}(E_{ij}-E_{i.})^2$. Since by Jensen's inequality $\mathrm{E}(1/X) > 1/\mathrm{E}(X)$ for positive random variables X, we might in principle expect a slight bias away from 0 when this correction factor is applied. A consistent estimate of the standard error can be obtained by multiplying the conventional estimate by the correction factor F. Table 15 gives results when the bias correction is applied. No remaining bias is noticeable.

6 Conclusions

The bidirectional case-crossover design is a valuable method for estimating acute effects of environmental exposures that takes advantage of two features of studies involving environmental exposures: (1) accurate information about past exposure is often available, so model-based estimates of exposure are not needed, and (2) levels of exposure are unaffected by the occurrence of failure of a subject. In contrast to other case-crossover designs, control information is assessed both before and after failure, which eliminates confounding due to time trends in exposure.

The proposed two-stage analysis of the multilevel analytic design provides asymptotically unbiased and efficient estimation of effects in a complex model involving unmeasured between-groups differences, measurement error, and a complex measurement model combining individual and aggregate exposure data. In particular, in cases where the within-groups exposure variance is less than the between-groups variance, estimates obtained through pooling can be more efficient than estimates based on either individual level or aggregate level analyses alone. Simulation techniques can be used to optimize the various trade-offs between the design parameters if reasonable estimates of the model parameters are available. Since variables are measured on the individual level, the model should be relatively free from nonadditivity bias, but could be subject to bias in situations where its assumptions are seriously violated. We believe this design and its associated analysis offers considerable promise for resolving some of the difficulties of between-group confounding, measurement error, and restricted variability that have historically plagued environmental epidemiology.

Our work on measurement error in exposure assessment represents an initial attempt to address some issues in the accuracy of estimated health effects of air pollution under various forms of exposure assessment. Refinement of the techniques used here will lead to more accurate results. It is quite clear from our work so far that neither the microenvironment nor the personal sampler method produces reliable estimates of exposure-response slope when measurement error is uncorrected. In our examples, the bias was toward 0 under the assumptions of our model, because of non-differential measurement error. If the measurement error were to involve bias that was correlated with the response, a bias away from 0 could result as well.

Our simulation studies show that the standard error of the estimate of a health effect increases as the errors in exposure assessment increase. When the fraction of the ambient level in a microenvironment is estimated with a standard error of 30%, the standard error of the estimate is 50% higher than it would be if the true exposures were known. Interestingly, it appears that errors in estimating indoor/outdoor ratios have much more influence on the accuracy of the microenvironmental approach than do errors in estimating the time spent in the microenvironments.

In order to adjust for measurement error, something about its distribution must be known. To correct the bias in $\hat{\beta}$, the variance (and bias, if any) in the measurement error must be known. Because of the great impact of measurement error, it is clear that careful validation studies are necessary before exposure assessment methods can be relied on to provide accurate estimates of health effects of pollutants.

7 Acknowledgments

We would like to thank Dr. Aaron Cohen of HEI for his unflagging encouragement and support of this project. Dr. Cohen led us to find many fruitful avenues of research that otherwise would have gone unexplored. This project owes a lot to his inspiration. We also appreciate the efforts of Dr. Geoffrey Sunshine of HEI, who assisted us in writing this report and made valuable suggestions regarding its organization. Ms. Virgi Hepner of HEI developed creative solutions to the many problems involved with getting the manuscript into print, which enabled us to present our findings in our own style.

Several of our colleagues on the USC Children's Health Study research team were instrumental in the success of our work. Dr. John Peters helped us put the data in a realistic context, and his knowledge of the epidemiology of respiratory disease enabled us to formulate more realistic statistical models. Fred Lurmann provided expertise on exposure assessment that lies behind much of the work in section 5. Ed Avol provided us with much background knowledge of air pollution and pulmonary function assessment that enabled us to formulate our results in ways more relevant to actual practice.

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10 Publications Resulting from this Research

Navidi, W., Thomas, D., Stram, D., and Peters, J. (1994). Design and analysis of multilevel analytic studies with applications to a study of air pollution. Environmental Health Perspectives 102, (Suppl 8), pp 25–32.

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11 Abbreviations

α_g	baseline outcome for group g
β	increase in log odds of failure due to a one-unit increase in exposure
$\hat{\beta}$	an estimate (e.g., maximum likelihood estimate) of β
$\hat{\beta}_{1-1}$	bidirectional case-crossover estimator with a single control day for each subject randomly chosen from among all control days
$\hat{\beta}_{BI}$	bidirectional case-crossover estimator using the full stratum of control days for each subject
$\hat{\beta}_{lag14}$	conventional case-crossover estimator with control times 14 days before failure
$\hat{\beta}_{lag28}$	same as above, with control times 28 days before failure
$\hat{\beta}_{TH}$	total history version of case-crossover estimator in which all days prior to failure are used as controls
λ_i	baseline log odds for subject i
A_i	set of days on which subject i is absent
D_{n_i}	the collection of all sets of n_i days
E	expectation
$I(\beta)$	Fisher information, used to estimate the variance of the maximum likelihood estimator $\hat{\beta}$
$L(\beta)$	log likelihood
N_{ij}	number of subjects at risk in community i on day j
n_i	number of days that subject i is absent
p_{ij}	probability that subject i is absent on day j
PM_{10}	particulate matter smaller than 10 microns in diameter
RMSE	root mean square error
S	arbitrary set containing n_i days
SD	standard deviation
SE	standard error
$t_1, ..., t_M$	list of days a subject is at risk
v_{gi}	confounder variable for subject i in group g
X_g	mean exposure for group g
X_{ij}	exposure of subject i on day j
x_{gi}	exposure variable for subject i in group g
y_{gi}	outcome variable for subject i in group g

INTRODUCTION

The Health Effects Institute (HEI)* has had a long-standing interest in the long-term effects of air pollutants on human health. In 1991, HEI set up the Environmental Epidemiology Planning Project to identify key methodological issues that needed to be addressed in the design and interpretation of future environmental epidemiologic studies of the health effects of air pollutants. Leading scientists identified several major concerns, including the relatively low levels of air pollutants to which populations are generally exposed and the consequent difficulty in establishing health risk; the difficulties inherent in measuring exposure to different pollutants; and the frequently high correlation among different pollutants, which makes it difficult to identify the effect of a particular agent. At the outcome of the Environmental Epidemiology Planning Project, further research on the design, measurement, data analysis, and interpretation of epidemiologic studies was recommended (Morgenstern and Thomas 1994; Hatch and Thomas 1994; Prentice and Thomas 1994). To meet these research needs, HEI issued Request for Applications (RFA) 91-1, *Epidemiological Studies of the Health Effects of Long-Term Ozone Exposure*, in 1991. The major goals of the RFA were to support (1) the development of epidemiologic methods for studying the effects of long-term ozone exposure, and (2) the development and testing in actual data sets of innovative approaches to the control of confounding of ozone effect measurements by other air pollutants.

In response to the RFA, Dr. Navidi and colleagues at the University of Southern California (USC) submitted a proposal entitled "Statistical Methods for Epidemiological Studies of the Health Effects of Air Pollution." The goal of the proposed study was to develop statistical designs to provide efficient estimates of the health effects of exposure to air pollutants in epidemiologic studies. As part of their research plan, Navidi and colleagues also intended to evaluate the effects of measurement error in exposure assessment (that is, the difference between true and estimated exposures) on the accuracy of estimated health effects. This is an area of critical interest, because problems in accurately measuring an individual's exposure may result in uncertainty in assessing the health risk of an air pollutant.

The HEI Research Committee, commending the caliber of the study team and the quality of the proposed methods, approved funding. In addition, they noted that a key strength of Navidi's application was that the statistical designs he developed would be applied in an ongoing cohort study conducted by a team of investigators at USC. This ten-year study, *Epidemiologic Investigation to Identify Chronic Health Effects of Ambient Air Pollutants in Southern California*, referred to as the "USC Children's Health Study" and sponsored by the California Air Resources Board (CARB), seeks to determine the health effects of long-term exposure to ozone and other air pollutants. Approximately 3,600 children from twelve communities in the southern California air basin exposed to differing components and levels of air pollutants are involved. The USC investigators, led by Dr. John Peters, are examining a variety of health endpoints, including changes in pulmonary function, days of school absence, and the appearance or augmentation of clinical symptoms, in relation to long-term exposure to ozone, particulate matter, nitrogen oxides, and acid (H^+). As described in the accompanying Investigators' Report and in subsequent sections of this Commentary, Dr. Navidi tested several of his epidemiologic models with data from the USC Children's Health Study.

Navidi's was one of six studies funded under RFA 91-1; four of the other five have already been published by HEI (Loomis et al. 1996; Avol et al. 1998; Kinney et al. 1998; Tager et al. 1998a,b). During the review of the study, the HEI Review Committee and the investigators exchanged comments and clarified issues in the Investigators' Report and in the HEI Review Committee's Commentary. This Commentary is intended to aid HEI sponsors and the public by highlighting the strengths of the study, pointing out alternative interpretations, and placing the report into scientific perspective.

BACKGROUND AND AIMS

The investigators begin their report by noting that there are three fundamental challenges common to many environmental epidemiologic studies: measuring exposure, dis-

* A list of abbreviations appears at the end of the Investigators' Report.

Dr. William Navidi's 3-year study, *Statistical Methods for Epidemiologic Studies of the Health Effects of Air Pollution*, began in March 1993 and had total expenditures of $285,915. The Investigators' Report from Dr. Navidi and colleagues was received for review in December 1996. A revised report, received in July 1997, was accepted for publication in October 1997. During the review process, the HEI Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Commentary.

ease, and related variables; finding populations with different degrees of exposure that are comparable with respect to potentially confounding factors; and distinguishing effects at the individual level.

Dr. Navidi and his colleagues undertook a study with three specific aims to address the methodological implications of these challenges. The aims were

1. to develop efficient statistical designs for panel studies,
2. to develop methods that allowed for the effects of exposure measurement error, and
3. to develop methods for combining individual and aggregate level comparisons.

The investigators developed a bidirectional case-crossover design to address the first aim. They addressed the second aim by developing mathematical measurement error models to compare community measures of exposure (termed "microenvironmental" or "indirect" by Navidi) with the direct, "personal sampler" approach. They also used this model to assess the impact of measurement errors. Finally, they developed multilevel analytic designs to meet the third aim. For each statistical model and design the investigators used the USC Children's Health Study data for illustration. These three approaches are discussed in the subsequent sections.

BIDIRECTIONAL CASE-CROSSOVER APPROACH

The case-crossover design (Maclure 1991) is a statistical design in which only cases are studied, and their exposure at the time of the event of interest is compared with some estimate of their typical level of exposure. This design is an adaptation of a case-control design and a crossover design. In a study utilizing a case-control design, cases (that is, subjects with a certain condition, for example, absence from school) are compared with controls (for example, subjects not absent). In a crossover study, subjects are compared under two different conditions (for example, when absent and when not absent from school). The case-crossover design blends the two approaches. The same subject is both a case and a control, and "crosses over" from being a case to being a control. That is, the health status of the subject is observed both when the patient is absent from school (case status) and when the subject is not absent (control status).

In this health effects study utilizing a case-crossover design, pollutant exposure when a subject is a case (is absent from school) was compared with the "typical" exposure when the subject is a control (is not absent). Further, in the usual case-crossover setting, the case data (exposure during time interval of case) is compared to some estimate of a previous control setting (exposure during prior time periods) when the individual is a noncase. For this example, the pollutant exposure when a child is absent from school is compared to some usual estimate of prior exposure when the child is not absent from school. For environmental studies, use of this previous time period control exposure data can introduce a number of serious problems and biases. In particular, the control period exposure data may be systematically lower or higher than the case period exposure data (possibly related to time trends) (see Greenland 1996).

As the investigators noted, the case-crossover design has been introduced and investigated by a number of others in the social sciences (Maclure 1991; Feldman 1993a,b; Mittleman et al. 1995). One useful contribution of Navidi and colleagues comes from understanding and applying the method in the air pollution exposure setting.

The case-crossover method is often used to investigate if some recent behavior has triggered or is related to the cause of an event (for example, in Marshall and Jackson [1993], the objective was to investigate whether the onset of a myocardial infarction [MI] was related to recent alcohol consumption). The method has at least two problems, which may lead to biased results: (1) it is very sensitive to the mathematical model employed, and (2) the control period data often used in the analysis may not be "typical" and so may not be good control data. In addition, as with the application of any epidemiologic method to observational data, obtaining statistically significant associations does not establish causality. Establishing causality usually requires synthesizing a wide range of evidence obtained using a number of approaches.

THE INVESTIGATORS' APPROACH

The investigators addressed the problem of biases in case-crossover studies by developing a bidirectional case-crossover where control exposure data were obtained both before and after the subject was a case (or, in the vocabulary of the investigators, before and after the subject was a failure). In addition, the investigators advocated using observed (recorded) exposure data for the control data rather than some estimated typical exposure.

The investigators conducted two simulation studies to evaluate the standard case-crossover design and their bidirectional case-crossover. For this particular model, they demonstrated bias in the standard case-crossover design and the lack of bias in their bidirectional method.

Lastly, they tested the bidirectional case-crossover approach by relating the acute effects of exposure to air pollution to absences from school for the USC Children's Health Study. The pollutants considered were ozone, particulate matter with a diameter less than 10 micrometers,

and nitrogen dioxide (NO_2). The analysis controlled for day of the week, temperature, and humidity. Because the subject acted as his or her own control, the analysis automatically controlled for subject-specific variables such as sex, age, race, community of residence, and prior health conditions. The analysis suggested that a relation between school absences and exposure to NO_2 lagged by two days. It should be noted that this effect was the only statistically significant one of many comparisons.

CRITIQUE OF THE BIDIRECTIONAL CASE-CROSSOVER METHOD

Have Navidi and colleagues eliminated or diminished the problems of the case-crossover by introducing the bidirectional aspect (that is, by using control data from both before and after the case period)? In the example they cite (absence from school in the USC Children's Health Study), the bias appears to be reduced. This is due to more appropriate control data. The issue of sensitivity to the mathematical model has not been resolved, however, because they did not investigate this issue.

In addition, the application of the bidirectional method may be in question for endpoints more extreme than absence from school. For example, in trying to employ the method for investigating the relation of pollutant exposure to MI, the subject may be extremely different before the event than after it. Thus, the properties of the subject in the control periods before and after the MI may be different. In the standard crossover design this would be related to what is called a "carryover effect": the MI changes the person. The current version of the bidirectional case-crossover design does not appear to accommodate this possibility.

In summary, the bidirectional case-crossover is an advance over the case-crossover design and shows the potential bias in the latter. The bidirectional case-crossover can incorporate better control data into the analysis. It needs investigation to study the sensitivity to the mathematical model, however, including carryover effects.

MEASUREMENT ERRORS IN AIR POLLUTION EXPOSURE ASSESSMENT

In this phase of the project, the investigators studied two ways of measuring cumulative exposure, the indirect or "microenvironmental" approach and the direct or "personal sampler" approach. The microenvironmental approach estimates the time spent in each microenvironment and also estimates an exposure time to each pollutant in each microenvironment. From these estimates a time-weighted average exposure for an individual is determined. The personal sampler approach requires that an individual wear a sampling device. The individual's average exposure is then estimated from the integrated concentration measured by the sampler and the amount of time the sampler was worn.

The two approaches differ in significant ways. With the microenvironmental approach, the exposure metric is flexible—exposure could be estimated as peak exposure above a certain level or as a cumulative exposure. In the personal sampling approach, only the cumulative exposure value is available. A second important difference involves the number of parameters to be estimated. The microenvironmental method requires estimates of both concentration of the pollutant and length of time spent in each environment; the only uncertainty in the personal sampler method is in the concentration, since the amount of time spent in each microenvironment is always correct.

The investigators used a simple model for the true exposure and compared the results from the two approaches under a variety of assumptions about the magnitude of the measurement errors involved. They performed a simulation study varying the measurement errors. Their conclusion was that neither the personal sampler nor the microenvironmental model gave correct answers when uncorrected measurement errors were present.

CRITIQUE OF MEASUREMENT ERRORS IN AIR POLLUTION EXPOSURE ASSESSMENT

Again, this component of the study was a careful, well-presented analysis. The investigators' conclusion that reliable results will not be achieved when there are measurement errors was based on a simple model. The investigators believe that more elaborate models can be constructed that will be self-correcting, and this will result in reliable estimates of cumulative exposures, but it is not apparent how this will be done. In addition, given the lack of literature concerning the validation of microenvironmentally based methods of exposure assessment and the limitations of the personal sampling methods to estimate cumulative exposure that Navidi and colleagues document so well, it is not clear how the comparison results should be viewed. More basic research is needed to determine whether the two approaches (microenvironmental and personal samplers) will be capable of estimating cumulative exposures accurately, and, if so, how well. Further, more extensive modeling and simulations are still needed to make any definite conclusions.

MULTILEVEL ANALYTIC DESIGN

To address the third aim of the project, to develop methods for combining individual and aggregate level comparisons, the investigators propose multilevel analytic designs.

In epidemiology there are two general types of studies for obtaining data to quantify the association between an exposure and a disease: ecologic studies and analytic studies. In the former, disease rates in groups of individuals are related to the average exposure rates in the groups. In the latter, the individual's disease outcomes are related to his or her own exposure values. Cohort and case-control studies are examples of analytic studies.

Often the results of ecologic and analytic studies are different. The resolution of the differences usually rests on three issues:

1. between-group confounding: a characteristic of groups that is not accounted for in the model but is a real risk factor. The outcome of interest may occur not as a result of the exposure, but instead as a result of some other group characteristics, including differences in medical diagnosis and reporting.
2. measurement error: the inability to measure without error the exposure variable(s). In analytic studies, for example, effects (the quantification of the impact of an exposure on an outcome) may be diluted due to measurement errors.
3. restricted variability: this results when, for example, a study is conducted on only one set of subjects and there may not be a wide enough variability in an exposure variable to reveal a true association.

Ecologic studies are often less expensive than analytic studies, are less prone to measurement error, and usually have good variation across groups. They may, however, not be well suited for the control of confounders. Analytic studies are usually better able to deal with confounders, and usually have better quality data, but may suffer more from measurement errors. Both of these types of studies are important, and they may often complement each other.

THE INVESTIGATORS' MODEL

The investigators suggest a multilevel analytic design where a number of groups are selected and within each group a sample of subjects is drawn. The analysis can incorporate variables at the subject level and also at the group level. This design incorporates the positive features of the ecological and analytic studies while addressing the deficiencies.

Their model, given as Equation 12 in the Investigators' Report, is as follows:

$$y_{gi} = \alpha_g + \beta x_{gi} + \gamma \upsilon_{gi} + \varepsilon_{gi}$$

where y_{gi} is the outcome variable for individual i in group g (for example, a lung function test value for a child in community g); and α_g is the average of the outcome variable for group g, which may be fixed or random effect. In this context, a fixed effect would mean that the groups in the study are the only groups of interest, while a random effect would mean that the communities in the study represent a random sample of some bigger collection of groups. The variable x is the exposure or exposures of interest, υ represents the set of confounders, and ε are the error terms. The confounders may be fixed or random. Often the ε are assumed to be normally distributed. The main interest lies in estimating β, the regression coefficient for the exposures x.

The investigators discussed weighted least squares and two-stage least squares as methods for estimating the model regression coefficients. In addition to the random effect models, the investigators incorporated two exposure measurement error models into their multilevel model. The Berkson model assigns individuals the group exposure (Armstrong 1990); the classical model assigns a random value whose expected value is the true exposure (Thomas et al. 1993). In this current work, they attempted to estimate the true effects of exposure (variables ε of the above model) because of the impossibility of measuring ε without error.

The investigators examined some "optimal" properties of their models as they related to the number of groups and the sample sizes within groups. These were performed under simplifying assumptions. An example was given from the USC Children's Health Study, which involved approximately 3,600 children from 12 southern California communities who underwent spirometric assessment of pulmonary function. The investigated relation was between a measurement of lung function, FEV_1, and lifetime exposure to ozone.

CRITIQUE OF THE MULTILEVEL ANALYTIC DESIGN

The designs that the investigators propose are not new, but have been used for several years in education, economics, and other social sciences. Currently they are employed extensively in health-related fields. These methods have undergone, and continue to undergo, intense development and extensions and are known under headings such as "multilevel models" (Goldstein 1995), "random effects models" (Chapter 2 of Goldstein 1995; Searle et al. 1992), and "hierarchical models" (Bryk and Ravdenbush 1992). Von Korff and colleagues (1992) recommended their use in epidemiology, and they have been used in some air pollution studies. In addition, the estimation procedures (weighted least squares and two-stage estimates of a [â] and measurement error models) are standard for these problems.

As described in the critique of the case-crossover design, the main contribution of Navidi and colleagues has been to apply these methods appropriately to data on the health

effects of air pollution. The investigators present a useful advance in this field by identifying in detail the components needed to model the phenomena and by supplying reasonable mathematical models. Further, they provide appropriate ways of obtaining efficient estimates of the relevant model parameters that quantify the effect of the exposure variables in the models.

There are concerns, however. The multilevel models and measurement errors models are sensitive to model assumptions. The investigators present neither discussions about the sensitivity of the models to assumptions nor diagnostics for assessing the fit (or appropriateness) of their models. For example, the assumption of random uncorrelated errors is an important component of the models. This may be unrealistic in practice and may limit the applicability of these models to air pollution studies. Further, if the model is not valid, its application may result in serious bias. The mathematical sophistication of the models does not ensure applicability. The investigators have presented the models very well. Correct applications of these models will have to address the above concerns.

Last, the discussion of bias (in Section 4.3) is incomplete and does not relate directly, as the investigators state, to the bias discussed by Greenland and Robins (1994). The problem the investigators call bias relates to how interactions at the individual level (Equation 16 in the Investigators' Report) could bias main effects estimates for the ecologic analysis. Greenland and Morgenstern (1989) deal with this, as do Greenland and Robins (1994). They go on, however, to describe how nonlinearities in confounder dose-response at the individual level could bias exposure main-effect estimates in the ecologic analysis, even if there are no interactions at all, and even if the exposure effects are only linear. At a minimum, major simulation studies are needed to evaluate the effects of nonlinearities on the results of analyses from the investigators' model, referred to as Equation 12 in the Investigators' Report and in the previous section of the Commentary.

FINAL COMMENTS

The investigators' main contribution has been not so much in the development of new methods as in transporting and extending existing methods to the air pollution exposure field. Their simulations demonstrate the potential for bias in the usual case-crossover method and sound a cautionary warning to any who desire to use it. Their extension to the bidirectional case-crossover study design offers promise for reducing bias. In addition, the investigators' discussion of measurement error in air pollution exposure, although simple, does document the impact of measurement error of estimates of cumulative exposures.

Finally, the application of the multilevel model to exposure problems also offers potential for advancement. There are major concerns, however, related to model appropriateness and validation that need to be addressed before it can be recommended for general use. The use of these models will always require a careful consideration of the appropriateness of the assumptions made and an understanding of potential biases inherent in the models.

In general, Navidi and his collaborators have made advances in statistical methodology, but have also, as is to be expected, left some issues unresolved. Applications of their methods to exposure problems, which include careful model development and validation, are needed to resolve these questions.

ACKNOWLEDGMENTS

The Health Review Committee would like to thank the ad hoc reviewers for their help in evaluating the Investigators' Report; Dr. Geoffrey Sunshine for his help in preparing the Commentary; and Thomas Atwood, Julia Campeti, Victoria Casana, Sean Donahue, Virgi Hepner, and Hope Steele for their roles in publishing this report.

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May 1999