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Project Summary

An Isotopic Study of the Inhalation Toxicology of Oxidants

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The purpose of these studies was to develop novel methods to investigate the biological fate of inhaled ozone and other oxygen-containing pollutants in animal and human tissues using the heavy isotope of oxygen, oxygen-18 (^{18}O). Methods were developed that facilitated the conversion of tissue oxygen to CO_2 and the subsequent trapping of the CO_2 so that it could be subjected to isotope-ratio mass spectrometry. The ratios of the various masses of evolved CO_2 were used to calculate the ^{18}O content of the original tissues, thus enabling the detection of isotopic enrichments as small as 0.4%. The above procedures were performed by modification of a commercially available elemental analyzer to include effluent columns and trapping devices, development of oxygen isotopic standards, and by derivation of mathematical models for correction of blank and memory effects originating during sample pyrolysis.

These procedures were applied to detecting reaction products of inhaled ozone ($^{18}O_3$), and in measuring tissue oxidation which occurs during exposure to the hepatotoxin, carbon tetrachloride. Mice which were exposed to 1 ppm $^{18}O_3$ showed measurable levels of ^{18}O in their lungs by 20 minutes of exposure, then increasing to higher levels as exposure was continued. The label was found in all biochemical fractions of the tissue: lipid, solute, and macromolecule. It was detectable in tracheal and nasopharyngeal tissue of mice but not in blood. Rats and rabbits also showed excess ^{18}O in their lungs following exposure to $^{18}O_3$, however, it was only possible to detect it in other tissues when excised epithelium of the nasal and tracheal airways was sampled. The levels of ^{18}O in the epithelium appeared to far exceed those seen in homogenates of the whole lung (on a per gram basis).

The experiments in which carbon tetrachloride was used demonstrated for the first time that lipid peroxidation is detectable *in vivo* as incorporation of oxygen-18. Rats exposed to an artificial air mixture made from $^{18}O_2$ showed substantial amounts of ^{18}O in the lipids as well as in the solute and macromolecular fractions of the whole tissue. The amount of ^{18}O incorporation appeared to be proportional to the activity of the cytochrome-P450 monooxygenase system which metabolizes the carbon tetrachloride.

These results confirm that ^{18}O tracing studies can be applied to at least two important problems in inhalation toxicology, and suggest the need for further studies in this area.

This Project Summary was developed by EPA's Health effects Research Laboratory, Research Triangle Park, NC, to announce key findings of the research projects that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Inroduction

Physiological tracing studies using oxygen have been severely limited by the short half lives of all radioactive isotopes of this atom. Since oxygen-17 and oxygen-18 are already present in all normal bio-

logical material, tracing studies must focus on detecting the excess over the natural abundance of these isotopes, which at lower levels of detectability requires the technique of isotope ratio mass-spectrometry. Oxygen-18 is the isotope which is most readily available commercially, therefore, it is most commonly used for tracing studies.

All techniques for tracing ^{18}O in biological material require purification of a molecule of known mass which contains oxygen that can be subjected to mass spectrometry. Earlier techniques which have been described for preparation of carbon dioxide from oxygen in organic material suffered from two problems. First, inaccuracies were encountered due to fractionation of oxygen isotopes between two or more intermediates in the conversion pathway. Second, interferences arose due to oxygen contamination. Techniques which have successfully overcome these problems have required a great deal of skilled manipulation and have been useful only with samples containing little or no contaminating elements besides carbon and hydrogen.

The present study developed an improved procedure for oxygen isotopic analysis of physiological samples which involves (1) pyrolysis of the dried tissue sample, (2) conversion of the oxygen-containing pyrolysis products to carbon monoxide, (3) oxidation of the carbon monoxide to carbon dioxide by iodine pentoxide, and (4) analysis of the carbon dioxide to determine ^{18}O content in the samples.

Specifically, the methods developed involved determining total oxygen and fractional abundance of ^{18}O in 1-2 mg samples of dried biological tissues. Samples were weighed into silver cups and placed in the sample head of an elemental analyzer (Carlo Erba Instruments).* They were pyrolyzed in a stream of helium where the oxygen was quantitatively converted to CO by passing through a column of Ni-coated carbon. The CO was separated from other gases chromatographically and quantified by the analyzer to determine the wt % of oxygen in the sample. The CO effluent from the elemental analyzer was captured and oxidized to CO_2 by a I_2O_5-containing column added to the instrument. The CO_2 was collected and purified cryogenically and the fractional abundance of ^{18}O was determined using an isotope-ratio mass spectrometer (Finnigan, Inc.). Although the possible masses of the CO_2 ranged from 44 to 49 atomic mass units, ion currents large enough to allow rapid measurement of oxygen species from tissue samples usually occurred only at masses 44, 45, and 46. Mathematical and theoretical models were employed to convert the isotope ratios to oxygen-18 fractional abundances, and corrections were made in the data to account for blank and memory effects arising during the analysis. Such corrections were made possible using oxygen isotopic standards which were also developed and improved as part of this project. These standards were prepared by exchange reactions of benzoic acid with water samples that differed in their isotopic composition. The benzoic acid could be either directly decarboxylated to CO_2 or passed through the elemental analyzer the same as a tissue sample. The use of benzoic acid standards made possible the determination of the correction parameters to be used for whole tissues.

*Mention of trademarks or commercial products does not constitute endorsement or recommendation for use.

Results

The above techniques were applied to detect O_3 reaction products in laboratory animals following exposure to 1.0 ppm $^{18}O_3$. Mice exposed to $^{18}O_3$ showed an increase in $^{18}O_3$ above the natural background level that became significant after 20 minutes of exposure. A 30-min exposure resulted in about 10 nmoles of O_3-derived oxygen in the total respiratory tract, of which 56% was present in the nasopharynx, 5% in the trachea, and 39% in the lungs. No increase in blood ^{18}O was detected. Lung tissue which was fractionated into lipid, macromolecules, and methanol-water soluble phases showed an approximately equal uptake of $^{18}O_3$-derived ^{18}O into the different fractions when expressed on a per dry weight basis. Preliminary studies to determine the persistence of the ^{18}O label in the lung following exposure to $^{18}O_3$ showed that ^{18}O content was diminished to near control values by 12 hr post-exposure.

Rats and rabbits exposed to $^{18}O_3$ showed about half the enrichment of ^{18}O in the lungs as did the mice, while enrichments in the head and blood were below the level of detectability of the assay. The low level of ^{18}O in the head of the larger species was believed to be the result of the large amounts of bone in the pulverized tissues which contained such large amounts of natural ^{18}O as to make detectability of small enrichments more difficult. In an attempt to eliminate the isotope dilution problem, freeze-drie epithelium from $^{18}O_3$-exposed rabbit was sampled, and levels of excess ^{18}(compared in different regions of the res piratory tract. Much higher enrichment: of ^{18}O were seen in the epithelial tissues indicating that O_3 reaction products ar mainly localized in the respiration linin layers. O_3-derived ^{18}O was most concen trated in the nasal passages and uppe tracheal epithelium, becoming less con centrated in the peripheral portions of th lung.

The other application that was made o the ^{18}O tracing techniques was in detec tion of tissue oxidation using a mode lipid peroxidation initiator, carbon tetra chloride (CCl_4). The involvement of oxida tion reactions in CCl_4 toxicity, particularl lipid peroxidation, has been reported b such indirect measures as formation o conjugated dienes, fluorescent pigments malonaldehyde, and volatile alkanes ir intoxicated tissues. However, direct in corporation of oxygen has not beer reported previously. The cytochrome P 450 monoxygenase system of the live normally oxidizes lipid-soluble xenobiot ics to more polar compounds, thus facili tating their excretion. However in the case of CCl_4, toxic metabolites formed b the cytochrome P-450 system initiate a variety of pathological reactions includ ing inhibition of lipoprotein secretion int the plasma, swelling of mitochondria and decreased enzyme activities and pro tein synthesis. Induction or inhibition o the cytochrome P-450 system by pheno barbital or piperonyl butoxide causes ar increase or decrease, respectively, in the toxic effects of CCl_4.

In order to test the hypothesis that bound oxygen could be detected in live during *in vivo* CCl_4 exposure, rats were injected with CCl_4 (lg/kg) and maintained for one hour on a closed respirator system in which they breathed an artificial air mixture containing 21% $^{18}O_2$ in N_2. Rats exposed to $^{18}O_2$ alone incorporated 650 nmoles of excess ^{18}O/g liver and CCl_4-treated animals exposed to $^{18}O_2$ had an excess ^{18}O of 1230 nmoles/g live which was further elevated to 198(nmoles/g by phenobarbital pretreatment. Rats pretreated with piperonyl butoxide showed excess ^{18}O similar to groups no treated with CCl_4. A significant portion o the excess ^{18}O was found in each of the major liver fractions examined: metha nol-water soluble, chloroform soluble and pellet. These results suggest tha CCl_4 initiates autooxidation of non-lipid as well as lipid constituents of liver.

Conclusions

A new technique for tracing oxygen-18 in whole animals and complex biological samples has been developed. The method involves quantitative conversion of tissue oxygen to carbon dioxide, and evaluation of the masses of carbon dioxide evolved to afford a measurement of ^{18}O content. These techniques have been applied with success to the determination of the biological fate of inhaled ozone, and to the measurement of tissue oxidation induced by a model peroxidation initiator, carbon tetrachloride.

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The complete report, entitled "An Isotopic Study of the Inhalation Toxicology of Oxidants," (Order No. PB 86-109 485/AS; Cost: $16.95, subject to change) will be available only from:

National Technical Information Service
5285 Port Royal Road
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The EPA Project Officer can be contacted at:

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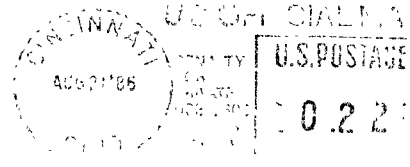

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