Table I— Concentration of Pilocarpine in the Aqueous Humor of 20- and 60-Day-Old Rabbits following Instillation of 25 μl of 1×10^{-2} *M* Pilocarpine

Minutes	Concentration[a], μg/ml	
	20-Day-Old Rabbits	60-Day-Old Rabbits
5	0.84 (0.03, 10)	0.55 (0.06, 8)
10	1.67 (0.05, 8)	1.03 (0.14, 11)
15	1.88 (0.17, 8)	1.11 (0.11, 8)
20	2.06 (0.10, 8)	0.98 (0.12, 10)
30	1.51 (0.18, 8)	0.93 (0.12, 9)
45	1.17 (0.12, 7)	0.42 (0.04, 8)
60	0.88 (0.12, 8)	0.44 (0.04, 7)
90	0.38 (0.03, 8)	0.19 (0.02, 7)
120	0.17 (0.01, 9)	0.11 (0.01, 8)

[a] Mean concentrations. The first number in parentheses refers to standard error, and the second number is the number of eyes sampled at that time point.

60-day-old male, New Zealand albino rabbits[1]. At various times postinstillation, rabbits were sacrificed and the aqueous humor was aspirated from the anterior chamber. Liquid scintillation counting allowed conversion of counts to micrograms of pilocarpine per milliliter of aqueous humor. All experimental procedures have been well established (3–5).

The aqueous humor concentration *versus* time profiles in the two categories of test animals are reported in Table I. At every time point, the concentration of pilocarpine was significantly higher in the 20-day-old rabbits as compared to the 60-day-old rabbits. The calculated areas under the curves were different by approximately a factor of two. The implication is that it should be possible to reduce substantially the dose administered to the 20-day-old rabbits while simultaneously maintaining aqueous humor concentrations equivalent to the 60-day-old rabbits. We make no comment, at this time, regarding the required concentration to produce a pharmacological effect.

Many studies currently appearing in the ophthalmic literature do not concern themselves to any extent with the age or size of animals used. These studies, although using relatively large differences in age, certainly point out the need for standardization of test animals. Also apparent are the inherent difficulties in comparing studies between laboratories without adequate knowledge concerning age and size of test subjects.

More importantly, further investigation into the development of ophthalmic pediatric dosage regimens is warranted. When one considers differences in the aqueous humor volume in the eye, as well as differences in the surface area available for absorption and the existence of immature membranes, it becomes apparent that some dosage adjustments may be in order, at least during the rapid growth phase of the eye (*i.e.*, birth to 3 years).

In addition, it is known that tear production and instilled volume drainage account for a large loss of drug from any topically applied dose (6, 7) and can affect the ocular bioavailability of drugs (8). No one to date has quantitatively considered these effects as applied to the bioavailability of topically administered drugs in children, nor have the potential toxic effects due to drainage been quantitated.

Also worthy of mention is the fact that numerous functional changes take place in the eyes of geriatrics. Decreased tear flow and volume are not uncommon with age and could cause differences in instilled drug concentration. One also might suspect changes to take place in the drainage of instilled solutions and, potentially, in the integrity of ocular membranes. All such changes have the potential to cause differences similar to those noted here and may warrant dosage adjustments.

Finally, one major problem in topical ophthalmic drug therapy of both infants and geriatrics is compliance with the medication dosage regimen. It is hoped that, by quantitating and maximizing dosage regimens, therapy can be simplified and the degree of compliance increased.

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[1] At the 20th postnatal day, the rabbit's globe is about two-thirds of adult size; at 60 days, the rabbit's globe attains 90% of adult size. The human eye is about two-thirds of adult size at birth and about 90% of adult size at 3 years of age.

Culture of Isotopically Substituted Plants of Pharmacological Importance: Conservation and Recycling of Stable Isotope Substrates

Keyphrases □ Isotopes, stable—^{2}H- and ^{13}C-substituted tobacco plants cultured, sealed growth chambers, isotope substrates conserved and recycled □ Plant culture—^{2}H- and ^{13}C-substituted tobacco plants cultured, sealed growth chambers, isotope substrates conserved and recycled □ Tobacco plants—*Nicotiana tabacum*, ^{2}H- and ^{13}C-substituted, cultured in sealed growth chambers, isotope substrates conserved and recycled

To the Editor:

We have successfully cultured algae, bacteria, protozoa, molds, yeasts, and fungi in fully deuterated form (1). Deuterated metabolites have been isolated from

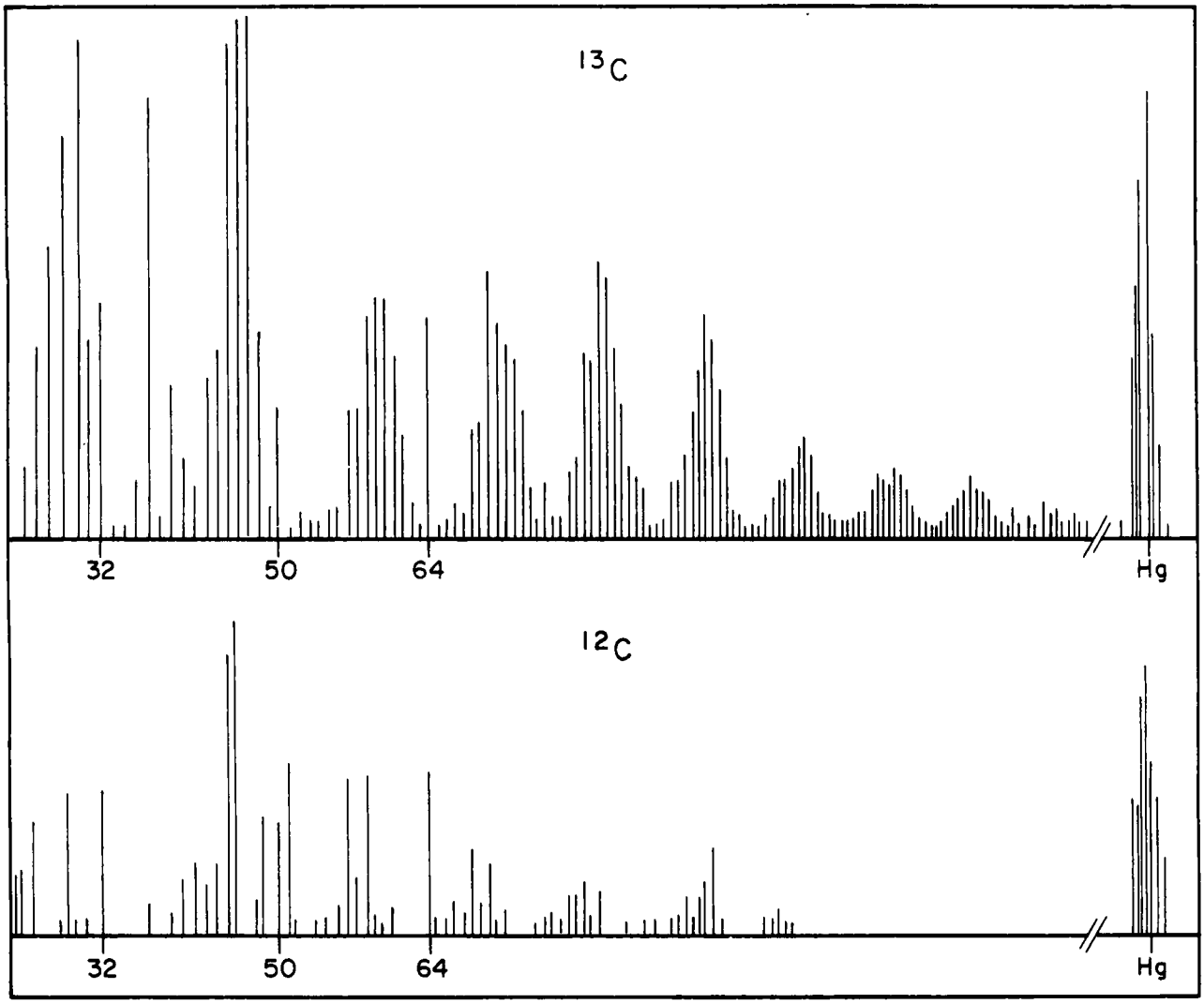

Figure 1—*Time-of-flight mass spectra of ordinary and carbon-13 white fly wing wax. The additional lines in the ^{13}C-spectrum arise from various combinations of carbon-12 and carbon-13 present in the wax hydrocarbons.*

these organisms and are being used in further studies. In culturing these organisms, deuterium oxide replaced the water in the usual nutrient medium. Where organic substrates were required, a serious effort was made to provide the deuterated analog to assure maximum deuteration.

Less successful have been attempts to culture higher plants in fully deuterated environments. Plant growth is severely inhibited when the deuterium oxide content of the growth medium exceeds 60%; at this time the possibility of obtaining more highly deuterated metabolites from higher plants seems remote. The cost of deuterium oxide is one limiting factor because large volumes of water must be used in such studies; with the facilities available, it has not been possible to recover and recycle the deuterium oxide from the spent nutrient medium or from the vapor transpired by the plants.

The results of our studies to date on pharmacologically significant substances were reported recently (2).

Our work in stable isotope biology has extended to studies of plant growth on highly enriched ^{13}C-carbon dioxide, the stable heavy isotopic form of carbon present in nature. The labile character of ^{13}C-carbon dioxide again makes for complications. The high cost of carbon-13 necessitates conservation in closed systems. We recently designed and tested completely sealed growth chambers suitable for culturing higher plants of pharmaceutical importance; rare isotopically substituted substrates (deuterium oxide and ^{13}C-carbon dioxide) are conserved during growth, and materials not utilized are recovered and recycled. Some features of the design and operation of these chambers were reported previously (3). We present here the results of experiments that illustrate the advantages of stable isotope studies with this type of growth chamber.

Three growth chambers are in operation in a climate-controlled greenhouse[1]. One chamber is always reserved for the growth of control plants using substrates of ordinary isotopic composition. These systems are of sufficient size (1 m high, 6 m^3 total volume) to accommodate large terrestrial plants. The chambers are completely sealed and leakproof, precluding loss of

[1] The authors are indebted to Dr. Norman R. Farnsworth and Dr. Harry H. S. Fong for permission to use the facilities of the University of Illinois Pharmacognosy and Horticulture Field Station, Lisle, Ill.

deuterium oxide or ^{13}C-carbon dioxide during the entire growth period (which may extend for several months or more) and assuring maximal and uniform incorporation of isotope.

The chambers are fabricated with an aluminum floor and structural elements and have a ceiling and walls of transparent acrylic sheets. A large circular port (covered by an O-ring sealed disk) located at one end of the chamber permits entry before and after a growth study. A bulkhead port is located at the opposite end of the chamber and provides for electrical power, gas, and liquid transport into or out of the chamber. Sets of glove ports, fitted with arm-length Neoprene dry box gloves, are located along both sides of the chamber and permit manipulations within the chamber during the period of plant growth.

Entry into the chamber and exposure to the external environment are completely avoided during the growth period of the plants under study. Temperature and humidity are controlled by continuous circulation of the chamber atmosphere over a heat exchanger. The heat exchanger is enclosed in a separate compartment extending into a plenum constructed along the ceiling. The highly humid atmosphere resulting from plant transpiration is directed from the growth area into the heat exchanger compartment, where it is cooled, and the dried gas is redistributed from the chamber top.

The condensed water is collected in a trough under the heat exchanger, passed through a mixed bed ion exchanger, stored in a reservoir, and conducted to a watering distribution system for recycling to the plant containers. Plants are watered periodically by means of a timer actuated by a solenoid valve, which permits water to be pumped to the distributor system at a predetermined rate. Soil moisture is monitored by conductivity probes embedded in the root support medium of each plant container.

In a typical growth study, seedlings are bedded in pots containing a vermiculite–sand mixture. For experiments with ^{13}C-carbon dioxide, this mixture ensured that there was no preformed carbon source available to the plants. In the deuterium oxide studies, the bed (*e.g.*, vermiculite or peat moss) was exchanged with the deuterium oxide solvent prior to the growth study to preclude dilution of the deuterium content of the solvent by the exogenous hydrogen of the potting material.

The carbon dioxide content of the atmosphere within the chamber was determined by measurement of the IR absorption of a 10-cm path length gas cell, calibrated for both ^{12}C-carbon dioxide and ^{13}C-carbon dioxide. In the deuterium studies, the deuterium oxide content of the nutrient was determined at the beginning and end of the study by comparing the density of the solvent with that of pure deuterium oxide (99.6%) and water (0% D_2O) at the same temperature.

Initial studies with these chambers involved the growth of tobacco[2] (*Nicotiana tabacum* L.) in an atmosphere of carbon dioxide highly enriched (90–92%) in ^{13}C-carbon dioxide. One objective was to observe any biological effects induced by incorporation of the heavy isotope of carbon by the plant during an entire life cycle of growth. Combustion of tobacco samples after harvest, followed by mass spectrometric determination of the carbon isotopes, indicated that the isotopic content of the plant material was essentially that of the carbon dioxide substrate employed during growth (about 90% ^{13}C).

A second objective of the study was to produce ^{13}C-enriched cured tobacco from the leaves. Reactive chemical species ("free radicals") are always present in tobacco smoke and are believed to be possible initiators of pathological conditions such as lung cancer. The presence of the heavy isotope of carbon in the free radical allows inferences to be made about the chemical nature of the molecular species from changes in the observed electron spin resonance spectrum. A study of the spectra of the smoke produced by the combustion of tobacco may aid in the identification of specific chemical species present in the smoke and possibly give insight into the nature of carcinogens present in tobacco smoke.

Other related studies involved the growth of tobacco in media containing 50 and 60% D_2O. In an earlier study (4), the effects of deuterium on tobacco plants were observed by growing the plants hydroponically and open to the air. This procedure required several hundred liters of deuterium oxide for only a few plants. For obvious economic reasons, it is generally not practical to grow plants in this manner on a large scale. Use of closed growth systems greatly reduces the amounts of deuterium oxide required for such an experiment.

We now have tobacco plants growing in deuterium oxide in two sealed growth chambers. The third chamber contains control plants. It is estimated that at maximum and complete utilization of growth space in one of these large chambers, using 70% D_2O levels of recycling solvent, the total volume of heavy water (99%) required for the entire experiment will be 30–35 liters. This amount is all that will be required to produce about 10 fully grown tobacco plants in a single chamber. Any residual deuterium oxide collected in the reservoir is, of course, available for recycling in other experiments. Another important consideration that recommends such closed systems is that very little attention to the plants is required after initial setup because of the complete automation of the system.

The growth chambers described here are, in effect, miniature ecosystems capable of supporting entire chains of isotopically substituted organisms that show metabolic dependence. One such relationship is that of host and parasite. In one series of ^{13}C-substituted tobacco experiments, after a growth period of several weeks, an infestation of white flies (*Trialeurodes vaporariorum*) was observed in the sealed chamber. This common greenhouse insect is parasitic on a wide range of plants and was evidently introduced adventitiously (as larvae or eggs) into the chamber at the outset of the experiment.

To prove conclusively that the mature insects had been using the isotopically substituted tobacco plants as their food source, the wing wax of the captured specimen was examined in a time-of-flight mass spec-

[2] A voucher specimen was deposited in the Chemistry Division, Argonne National Laboratory; the plants were identified by Mr. W. D. Atkinson, Department of Agronomy, University of Kentucky.

trometer[3]. The mass spectrum obtained (Fig. 1) contained additional mass peaks, one unit greater than the normal ^{12}C-containing peaks, in an abundance indicating massive carbon-13 incorporation into the constituents of the white fly tissue.

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Biased Bioavailability Estimates

Keyphrases □ Bioavailability—griseofulvin, use of fractional urinary excretion data questioned □ Griseofulvin—bioavailability, use of fractional urinary excretion data questioned □ Urinary excretion data, fractional—value in griseofulvin bioavailability studies questioned □ Antifungal agents—griseofulvin bioavailability, use of fractional urinary excretion data questioned

To the Editor:

In a recent communication (1), followed by publication of the detailed data (2), Bates and Sequeira proposed that 24-hr urinary excretion data of total (free and glucuronide conjugated) 6-desmethylgriseofulvin, the main metabolite of griseofulvin, be used to determine the bioavailability of the parent drug. However, I wish to point out that this general practice (1, 3, 4) of using fractional urinary drug excretion data and/or areas under the plasma level–time curve without proper pharmacokinetic justification, even with apparent correlations with total urinary excretion and total area under the plasma level–time curve data, is dangerous.

Table I—Relative Bioavailability of Formulations I–IV of Griseofulvin Calculated from the Ratios of the Total Urinary Metabolite at 24 and 96 hr[a]

Formulation[b]	24 hr	96 hr
I:IV	0.32	0.43
II:IV	0.41	0.53
III:IV	0.52	0.60
I:III	0.61	0.71
II:III	0.78	0.87
I:II	0.79	0.81

[a] Data are from Fig. 1 and Table II of Ref. 2. The differences among the relative bioavailabilities calculated from the urinary excretion data of total metabolite at 24 and 96 hr are statistically significant; two-tailed paired *t*-test: t (0.002) = 5.89, t calc = 5.97. [b] I = aqueous suspension, II = commercial Tablet A, III = commercial Tablet B, and IV = corn oil-in-water emulsion.

Bates and Sequeira (1) stated that, while the plasma levels of the entirely metabolized griseofulvin are usually low after oral administration of different formulations, the measurement of 24-hr urinary excretion of the main metabolite, 6-desmethylgriseofulvin, could be more reliable and more convenient since the use of a 72–96-hr urinary collection period "increases the chances for a lack of compliance to the experimental protocol on the part of subjects . . . ." These investigators reported that a correlation existed between the dose percentages of total 6-desmethylgriseofulvin excreted within 24 and 96 hr after administration of various formulations of griseofulvin and concluded that: "These correlations . . . provide the basis for possible utilization of 24-hr cumulative total 6-desmethylgriseofulvin excretion data as an index of griseofulvin bioavailability in humans."

However, an accurate assessment of the relative bioavailability of a given griseofulvin formulation from metabolite cumulative recovery excretion data measured over a limited 24-hr time interval is only possible if the absorption process of griseofulvin is complete much sooner than 24 hr after drug administration. However, with some oral formulations of griseofulvin, the absorption process occurs for 30–40 hr and even up to 80 hr (5). The bioavailability of such formulations would be underestimated by the use of 24-hr cumulative metabolite excretion data, since the total fraction of the dose administered that will eventually be absorbed is not totally absorbed at 24 hr after administration.

Urinary excretion data of the metabolite (free and glucuronide conjugated) after oral administration permit only the calculation of relative bioavailabilities of different formulations given by the same route of administration. Since griseofulvin is entirely metabolized (6) [most likely in the liver (7)], a first-pass effect, although not large (8–19% of the dose absorbed), must be anticipated (8). The linearity of an existing first-pass effect needs to be experimentally challenged. The fact that ultimately the amounts of free 6-desmethylgriseofulvin excreted in the urine are constant fractions of dosages of formulations with widely differing absorption characteristics does not negate a possible nonlinearity of griseofulvin metabolism (2). Parent drug data ob-