Application of a stable isotope (^{13}C)–labeled glycocholate breath test to diagnosis of bacterial overgrowth and ileal dysfunction

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The interval sampling of expired air offers a noninvasive methodology for the study of intestinal physiology. A GC-^{14}C breath test has wide clinical recognition as a test of intestinal bacterial overgrowth or ileal dysfunction. There is need for a nonradioactive, stable isotope analogue of this test, suitable for infants, children, and pregnant women, in whom the use of radioisotopes may be inappropriate. In the present study, stable isotope labeling and mass spectrometric analysis have been applied to the development of a GC deconjugation breath test using ^{13}C as the isotopic marker. In 13 adult subjects with a wide range of conditions associated with upper intestinal bacterial overgrowth or ileal dysfunction, the intraluminal deconjugation of standard glycocholate-1-^{14}C-glycine was compared to that of simultaneously administered glycocholate-1-^{13}C-glycine or glycocholate-1,2-^{13}C-glycine. The correlations of net deconjugation as measured by the radioactive isotope– and stable isotope–labeled bile salt was excellent (correlation coefficient 0.952). Exogenous bile salts did not affect the reproducibility of the breath test. There was statistical identity between the results of tests using glycocholate-1-^{13}C-glycine or glycocholate-1,2-^{13}C-glycine. The calculated detection limits employing a dose of 7.5 mg/kg body weight of glycocholate-1,2-^{13}C-glycine are 4.7% and 6.2% at the 95% and 99% confidence levels, respectively. This test offers potential advantages of safety and acceptability for infants, children, pregnant women, and women at risk of pregnancy.

Abbreviations: glycocholate (GC), thin-layer chromatography (TLC)

The ideal test of intestinal physiology in infants and children or in epidemiological field studies at the community level would be both noninvasive and nonradioactive. Breath tests,[1-4] in which samples of exhaled air are collected and analyzed, are a prime

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Portions of this work have appeared in abstract form[1, 2] and were presented in part at the annual meeting of the Central Society for Clinical Research, Chicago, Ill, November, 1975 and at the annual meeting of the American Federation for Clinical Research, Atlantic City, N J., May, 1976

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example of a noninvasive procedure. In recent years, a number of ^{14}C breath tests have come into clinical and investigative application. In 1971, Fromm and Hofmann[5] and Sherr et al.[6] reported a glycocholate-1-^{14}C-glycine* breath test as a noninvasive diagnostic procedure for ileal dysfunction of intestinal bacterial overgrowth. The test is based on the principle that the exposure of glycine-conjugated bile salts to bacterial enzymes from the intestinal flora, either as a result of abnormal overgrowth or as a result of failure of ileal absorption, results in cleavage of the molecule to glycine and cholic acid. The glycine is further metabolized to carbon dioxide by hepatic or bacterial enzymes. The rate and extent of CO_2 production from the isotopically labeled glycine moiety can be detected rapidly and quantitatively in the expired air.

^{14}C compounds, however, are of limited utility in children and pregnant women, and in populations at large, since there is a finite genetic and ecological hazard from the radioactivity. The use of analogous compounds, labeled with ^{13}C, a nonradioactive, stable isotope of carbon, would provide a safe alternative to radiocarbon tests. We present here the development, validation, and clinical application of a glycocholate breath test using ^{13}C-labeled material.

Methods

Patients. Of the 13 subjects studied (Table I), 12 were patients from the inpatient Gastroenterology Service or outpatient Gastroenterology Clinic of the University of Chicago Hospitals and Clinics; the remaining subject was a healthy member of the professional staff. The patients had conditions known or suspected to predispose to excessive bile salt deconjugation; in several, this had been determined by a previous GC-^{14}C breath test. The subjects ranged in age from 25 to 77 years (mean, 44 years). Six were male and seven were female. Weights ranged from 36 to 86 kg. The constancy of the $^{13}C:^{12}C$ isotope ratio of CO_2 in the absence of added ^{13}C was studied in an additional four healthy subjects. The experimental protocol and procedures were reviewed and approved by the Clinical Investigations Committee of the University of Chicago Hospitals and Clinics, and all studies were carried out with the written consent of the subject.

Synthesis of stable isotope compounds. Glycine was labeled with ^{13}C either in the 1-C position (carboxyl) or on both carboxyl and amino carbons, according to the method of Whaley et al.[7]

The glycocholate-^{13}C-glycines were prepared with 1,2-^{13}C-glycine or 1-^{13}C-glycine according to the method of Lack et al.[8] Product purity was determined by TLC on silica gel HF-254 (solvent system = ethyl acetate:isopropanol:propionic acid:water—40:20:30:10). The TLC plates were developed with 5% methanolic phosphomolybdic acid to visualize bile acids and 1% ninhydrin solution in acetone to visualize the amino acid, glycine. No amine-containing impurities were detected. A small impurity of cholic acid was detected and removed by recrystallization. Isotopic purity of the ^{13}C-labeled substrates was determined from the ^{13}C splitting of their proton nuclear magnetic spectra. The isotopic purity of both substrates was 90%.

Preparation of the ^{14}C compound. A 250 μCi quantity of commercially prepared glycocholate-1-^{14}C-glycine (Amersham-Searle Company, Amersham, England) with a specific activity of 50 μCi/mM was purified by TLC on silica gel H (solvent system = butanol:acetic acid:H_2O—10:1:1). A 10 μCi amount of the purified compound was dissolved in 1 ml of absolute ethanol as an individual dose and stored at −40° C until later use in conjunction with the ^{13}C compounds.

Administration of the simultaneous GC-^{13}C and GC-^{14}C breath tests. The subjects were studied after an overnight fast. Carbon dioxide in the expired breath was sampled 0.5 hr prior to the administration of the isotopically labeled substrates, at the time of administration, and at hourly intervals for 6 hours. The dose of 10 μCi of glycocholate-1-^{14}C-glycine and the dose of glycocholate-1-^{13}C-glycine or glycocholate-1,2-^{13}C-glycine (250 to 600 mg) were combined in a solution containing 1

*In previous publications, the following terms have been used to refer to the carbon-^{14}–labeled conjugated bile salt: cholyl-glycine-1-^{14}C; glycine-1-(^{14}C)–labeled glycocholic acid; 1-^{14}C-glycyl-cholic acid; glycine-1-(^{14}C)-glycocholate; glycine-1-(^{14}C) cholylglycine; cholyl-glycine-1-^{14}C; 1-^{14}C-glycyl-cholic acid, 1-^{14}C-glycine glycocholic acid; glycine-1-^{14}C-cholate. In the present paper the ^{14}C compound has been termed glycocholate-1-^{14}C-glycine; the ^{13}C compounds, glycocholate-1-^{13}C-glycine and glycocholate-1,2-^{13}C-glycine, respectively, as per IUB Committee of Editors of Biochemical Journals.

Table I. Simultaneous $^{14}C:^{13}C$ breath tests

Pt.	Sex	Age	Diagnosis	GC-^{13}C dose		Results*	
				mg/kg	mg	GC-^{14}C	GC-^{13}C
Glycocholate-1,2-^{13}C-glycine:							
S. F.	M	44	Crohn's disease, ileal resection	2.7	200	2.2	1.3
E. W.	M	77	Multiple jejunal diverticulosis	2.4	200	3.7	2.0
D. W.	F	29	Crohn's disease, ileal resection	5.0	200	1.3	0.5
D. M.	M	31	Postgastrectomy syndrome	3.5	200	0.1	0.0
L. B.	F	27	Diabetic gastroenteropathy	2.9	200	0.9	−0.1
B. J.	F	25	Crohn's disease, ileal resection	6.6	350	0.1	−0.1
M. G.	F	75	Jejunocolic fistula	8.1	350	3.3	1.7
Glycocholate-1-^{13}C-glycine:							
S. F.	M	44	Crohn's disease, ileal resection	7.5	560	2.4	1.2
E. W.	M	77	Multiple jejunal diverticulosis	7.5	550	4.0	2.4
F. F.	M	27	Crohn's disease, ileal resection	7.5	495	3.5	2.0
G. U.	F	30	Crohn's disease	7.5	270	0.0	0.3
M. C.	F	54	Ileorectal anastamosis	7.5	382	1.0	0.1
R. W.	F	35	Crohn's disease, ileal resection	7.5	360	2.6	1.3
R. B.	M	60	Crohn's disease, ?/enteric fistula	7.5	475	4.8	3.1
N. S.	M	30	Healthy control	7.0	600	0.0	0.6

*Expressed as (% administered dose/mM CO_2) × kg through 6 hr.

ml of ethanol and 10 to 20 ml of water and administered orally. Further oral intake for the duration of the test was limited to water ad libitum. For the collection of $^{14}CO_2$, exhaled breath was passed over anhydrous calcium sulfate into 1 ml of a 1M solution of Hyamine hydroxide (Packard Instrument Co., Inc., La Grange, Ill.) and 2 ml of absolute ethanol contained in a scintillation vial. Phenolphthalein was added as an indicator to ensure titration to neutrality, which represented collection of approximately 1 mmol of CO_2. For collection of $^{13}CO_2$, exhaled breath was passed through 10 ml of a 2N solution of bicarbonate-free sodium hydroxide (Dilut-it, J. T. Baker Chemical Co., Phillipsburg, N. J.) in a 250 ml round-bottomed flask for a precisely timed period of 5 min; approximately 10% to 30% of expired CO_2 was trapped as bicarbonate. On seven occasions, $^{13}CO_2$ was determined after administration of unlabeled glycocholic acid (Sigma Chemical Co., St. Louis, Mo.).

Quantitative measurement of $^{13}CO_2$. The technique used for the determination of $^{13}CO_2$ enrichment was that of Schoeller et al.[9] The dissolved carbon dioxide was released from solution in a vacuum by acidification with 20% H_2SO_4. The released CO_2 was collected in a −198° C trap (liquid nitrogen), first passing through a −78° C trap (Dry Ice–methanol) which removed most of the water vapor from the gas stream. The CO_2-containing trap was warmed to −78° C and the CO_2 was distilled into a second −198° C trap to further purify the sample. The ratio of $^{12}CO_2$ to $^{13}CO_2$ was determined for each sample on a dual collector isotope ratio mass spectrometer, and the excess ^{13}C was calculated from the increase in isotope ratio. The rate of production of $^{13}CO_2$ was calculated from the following formulas:

$$\% \text{ dose per mM } CO_2 = \frac{0.524\,(\delta a - \delta b)}{n \cdot D}$$

where b and a refer to before and after administration of the substrate, n equals the number of ^{13}C atoms per molecule in the substrate, D equals the dose of glycocholate in milligrams, and δ equals the per mil ^{13}C enrichment, as follows:

$$\delta = \frac{(^{13}C/^{12}C)\text{ sample} - (^{13}C/^{12}C)\text{ reference}}{(^{13}C/^{12}C)\text{ reference}} \times 10^3$$

The fraction 0.524 is a conversion factor to equate milligrams of glycocholate administered to the theoretically available millimoles of carbon dioxide. The excess $^{13}CO_2$ production for each time interval was taken to be the mean of the production at the beginning and the end of each time period. To

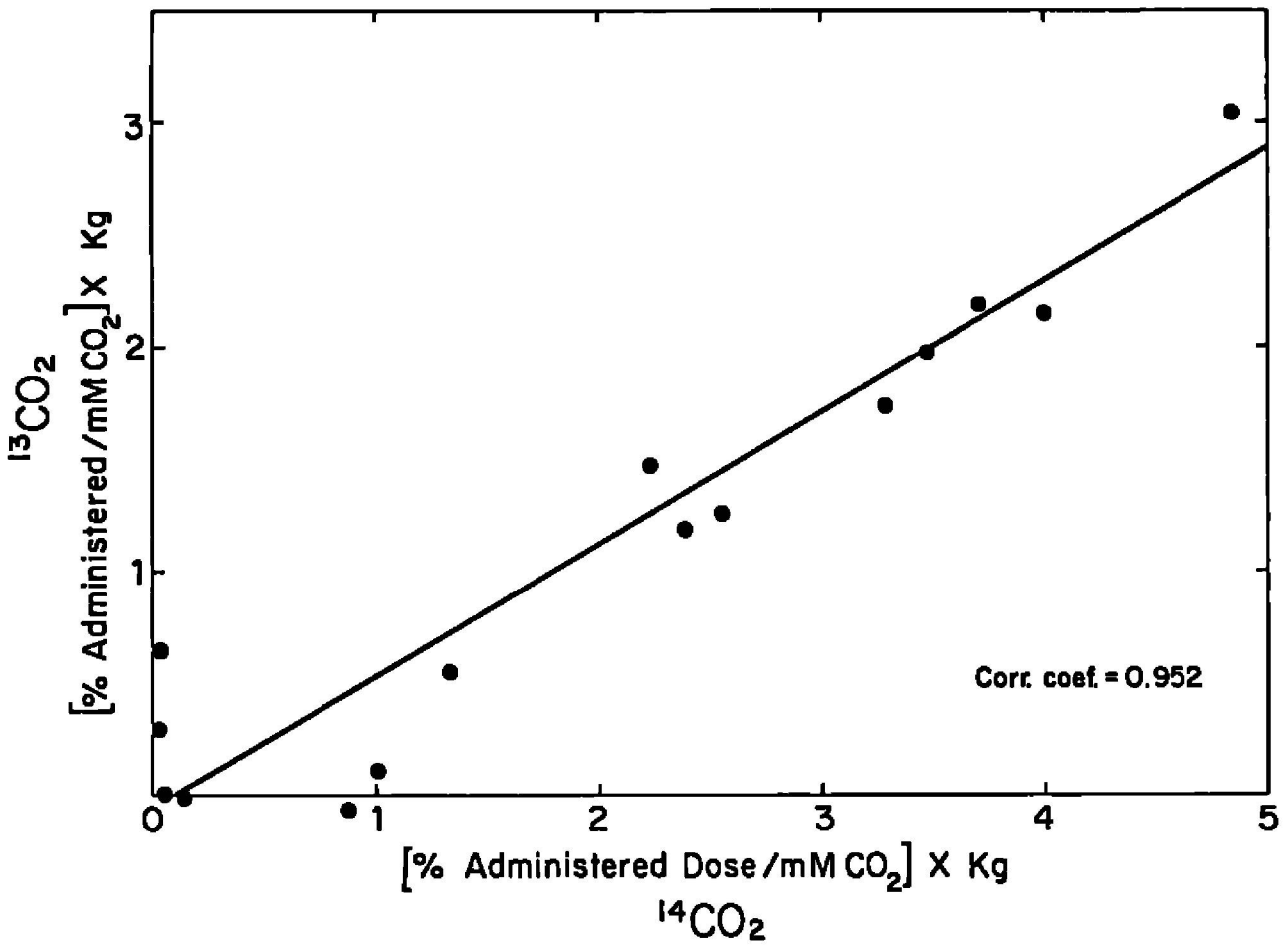

Fig. 1. Linear regression of 15 simultaneous GC-^{13}C and GC-^{14}C breath tests in 13 adult subjects.

allow comparison with the GC-^{14}C data, results were expressed as percent of administered dose times weight in kilograms per millimole of carbon dioxide.[5]

Quantitative measurement of $^{14}CO_2$. The exact quantity of carbon dioxide collected was calculated by titration of the Hyamine hydroxide solution with a standard solution of HCl. The beta radioactivity of the $^{14}CO_2$ was determined in a liquid scintillation counter (Iso-cap/300, Nuclear Chicago, Chicago, Ill.). The result for each sample was expressed as percent of the administered dose times weight in kilograms per millimole of carbon dioxide times 10^{-3}.[6] The $^{14}CO_2$ specific activity for each time interval was taken to be the arithmetic mean of the specific activity observed at the beginning and end of that time period. The cumulative excretion of $^{14}CO_2$ was calculated by multiplying the total $^{14}CO_2$ specific activity by the endogenous production of CO_2 (9 mM/kg/hr).

Statistical methods. Linear regressions were performed with the ^{14}C values as the independent variable and ^{13}C values as the dependent variable. Regression lines were compared in three steps by testing for homogeneity of the variances about the regression lines, acceptability to fit for parallel lines through the respective means, and absence of differences in elevation above the x axis.

Results

Baseline variability in $^{13}CO_2/_{12}CO_2$. To determine the baseline variation in the $^{13}CO_2 : {}^{12}CO_2$ ratio, carbon dioxide was collected in NaOH from seven adult subjects, three of whom were among the patients listed in Table I. Samples were collected before, and at hourly intervals for 6 hr after, the administration of unlabeled, synthetic glycocholic acid in the same milligram/kilogram dose as the subject would receive of the ^{13}C-labeled substrate. The doses ranged from 2.7 to 7.5 mg/kg. The variation in $^{13}CO_2$ abundance was ±0.74 ‰. Excess production of $^{13}CO_2$ after administration of the GC-^{13}C can be determined against this baseline variability.

Correlation of ^{13}C breath tests with ^{14}C breath tests. GC-^{13}C and GC-^{14}C breath tests were administered simultaneously in a total of 13 subjects on 15 occasions. The correlation between the ^{13}C and ^{14}C values was 0.952 (Fig. 1). The slope, however, was not the expected unity, but rather 0.59. The net 6 hr deconjugation as calculated for

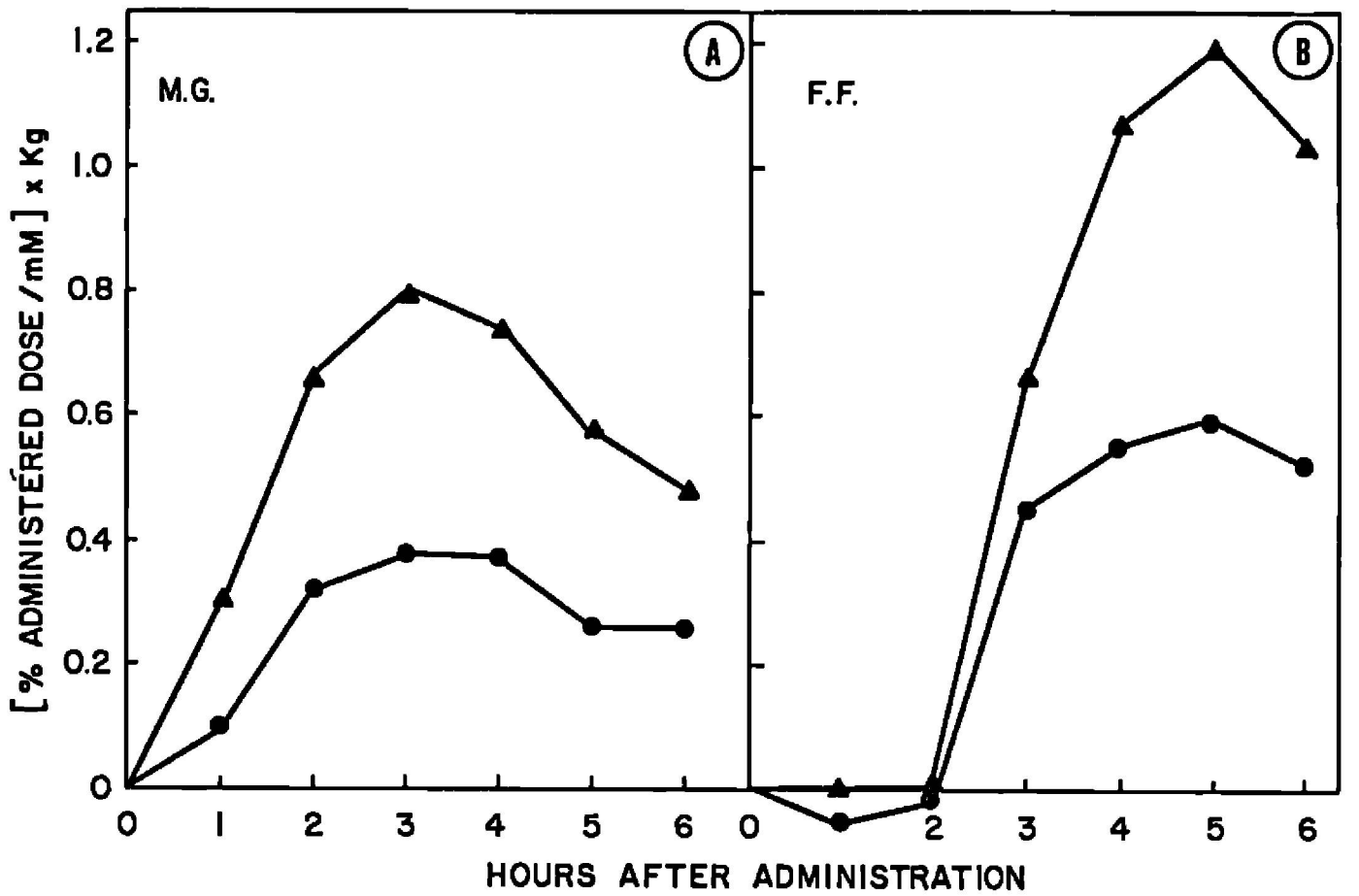

Fig. 2. Time course of simultaneous ^{13}C breath tests (circles) and ^{14}C breath tests (triangles) in two patients. *(A)*, Case of jejunocolonic fistula. *(B)*, Case of Crohn's disease after ileal resection.

glycocholate-1-^{13}C-glycine therefore was approximately 60% of that for the standard glycocholate-1-^{13}C-glycine breath test. There was no statistically significant difference between the behavior of the 1,2-^{13}C–and the 1-^{13}C–labeled substrates with regard to their relationship with the radiocarbon breath test.

Isotopic CO_2 excretion during simultaneous $^{13}CO_2$ and $^{14}CO_2$ breath tests. The pattern of isotopic CO_2 excretion as well as the net deconjugation was examined in the subjects after simultaneously administered oral doses of glycocholate-1-^{14}C-glycine and either glycocholate-1-^{13}C-glycine or -1,2-^{13}C-glycine. Fig. 2, *A*, shows the increase in specific activity of both $^{14}CO_2$ and $^{13}CO_2$ after administration of 10 μCi of glycocholate-1-^{14}C-glycine and 8.1 mg/kg of glycocholate-1,2-^{13}C-glycine to M. G., a 75-year-old woman with a jejunocolic fistula secondary to adenocarcinoma of the colon. Fig. 2. *B*, illustrates a similar plot after the administration of the standard dose of glycocholate-1-^{14}C-glycine and 7.5 mg/kg of glycocholate-1-^{13}C to F. F., a 27-year-old man with prior ileocolic resection for Crohn's disease. The point-by-point correlation between the specific activity of $^{13}CO_2$ and that of $^{14}CO_2$ had r values of 0.966 for M. G. and 0.984 for F. F.

Serial breath tests using first the glycocholate-1,2-^{13}C-glycine and then the glycocholate-1-^{13}C-glycine vs. glycocholate-1-^{14}C-glycine administered simultaneously were performed in two patients: S. F., a 44-year-old man with several previous ileocolonic resections for recurrent Crohn's disease, and E. W., a 77-year-old man with massive jejunal diverticulosis. Fig. 3 illustrates the studies on S. F. $^{14}CO_2$ has been expressed as the percent of the administered dose/mM of CO_2 times 10^{-3} and the $^{13}CO_2$ has been expressed as per mil change in abundance to correspond to the units of the baseline variability curve, which is included for visual comparison. Substantially equal values are seen for both the glycocholate-1-^{13}C-glycine– and the glycocholate-1,2-^{13}C-glycine–labeled bile salts; the corresponding GC-^{14}C deconjugation was found to be reproducible on the two occasions. Again the point-by-point correlation between isotopes is excellent, and the magnitude of

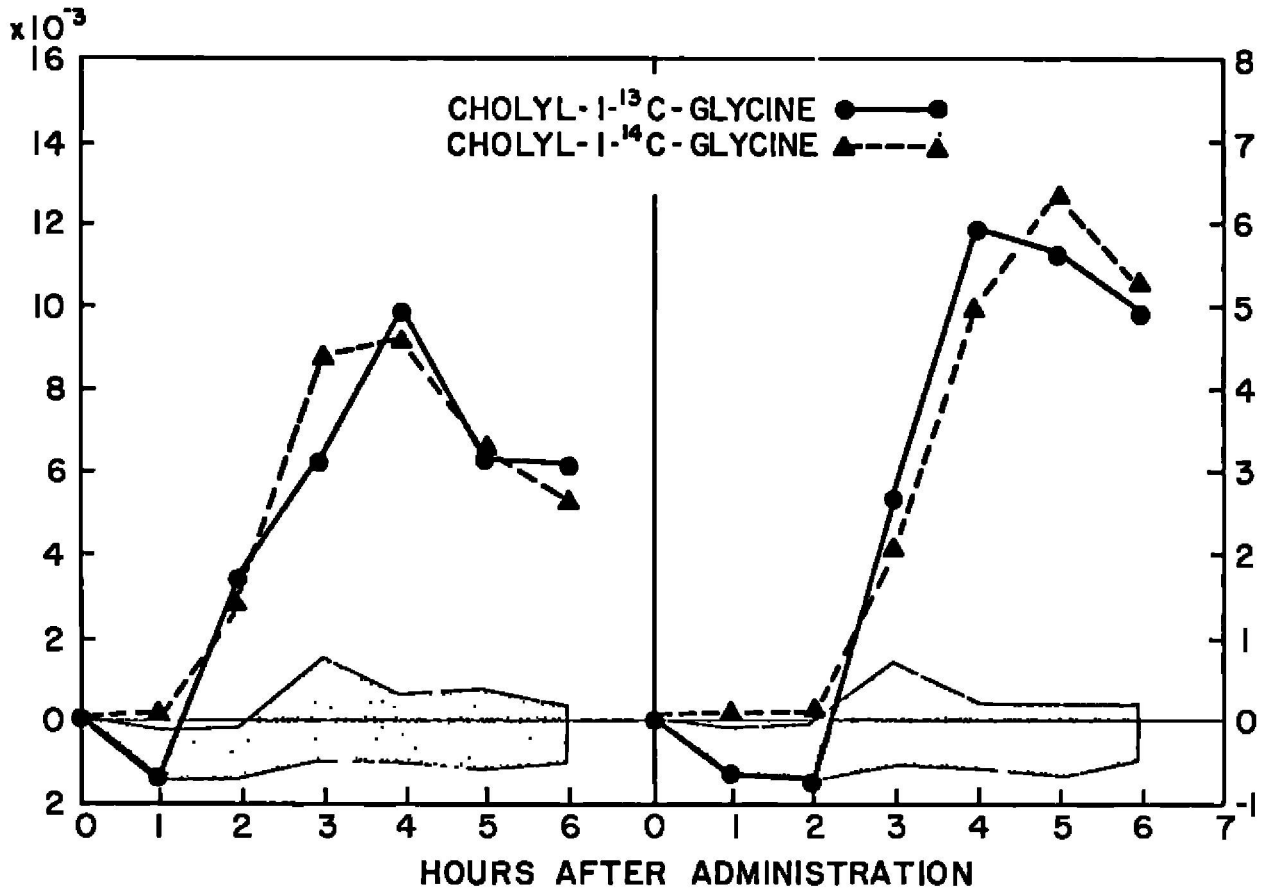

Fig. 3. Simultaneous ^{13}C breath tests (circles) and ^{14}C breath tests (triangles) on two occasions in the same patient, a man with Crohn's disease and extensive ileal resection. On the first occasion glycocholate-1,2-^{13}C-glycine was used; on the second, glycocholate-1-^{13}C-glycine. The $^{14}CO_2$ evolution is shown as change in specific activity. The $^{13}CO_2$ curve is measured in changes in $^{13}CO_2$ excess in parts per mil for comparison with the baseline variability shown in the shaded area.

the $^{13}CO_2$ signal above baseline is substantial (see below). A similar relationship between the three test substances was observed in the serial studies of E. W.

Sensitivity of the glycocholate-1-^{13}C breath tests. Evaluation of $^{13}CO_2$ breath tests has shown that their sensitivity is limited by the natural fluctuations in the isotope ratio of the endogenous CO_2.[9] These variations were shown to occur with a standard deviation which corresponds to 0.16 kg/hr % dose/mM CO_2 for the 6 hr test. When a dosage of 7.5 mg/kg of glycocholate-1,2-^{13}C-glycine is given, at a total CO_2 production rate of 9 mM/kg hr, this means that the 95% and 99% confidence limits for detection are 2.8% and 3.6% deconjugation, respectively. Compensating for the proportional response of glycocholate-1-^{13}C-glycine and glycocholate-1-^{14}C-glycine, the theoretical detection limits become 4.7% and 6.2%, respectively.

Effect of exogenous bile salts on the reproducibility of breath test results. In four of the 13 patients, glycocholate-1-^{14}C-glycine breath tests had been performed alone, either before or after the simultaneous ^{14}C:^{13}C breath test. This permits comparison of the effect of added bile salt on the breath test. As shown in Table II, there was less than a 3% difference in the net deconjugation calculated for GC-^{14}C breath tests administered alone or administered with relatively large doses of ^{13}C-labeled bile salt. Presumably, doses up to 0.56 gm of exogenous bile salt do not alter the response of the ^{14}C breath test.

Adverse reactions and side effects. The ^{13}C bile salt solutions in doses up to 600 mg were generally well tolerated by the subjects. Many patients complained of a pungent, bitter taste of the solution which lasted up to half an hour after administration. Only one patient, R. B., a 60-year-old man with short bowel, following multiple small bowel resections for Crohn's disease, experienced severe diarrhea and abdominal cramping following administration of 473 mg of GC. Symptoms resolved without therapy after 24 hr.

Table II. Effect of exogenous ^{13}C bile salt load on the reproducibility of the GC-^{14}C breath test

Pt.	^{14}C test alone (% adm. dose/mM $CO_2 \times 10^{-3}$)	Simultaneous ^{14}C:^{13}C breath tests	
		% adm. dose/mM $CO_2 \times 10^{-3}$	mg of bile salt
E. W.	56.4	54.6	550*
S. F.	29.6	30.3	200†
		32.1	560*
L. B.	11.7	12.9	200†
R. B.	74.9	76.9	472*

*Glycocholate-1-^{13}C-glycine.
†Glycocholate-1,2-^{13}C-glycine.

Discussion

Three major methodological problems are encountered when working with the stable isotope of carbon. These are the high background of 1.1% due to the natural abundance of ^{13}C, the variation of up to 25‰ in the isotopic content of carbon compounds from differing dietary sources, and the potential fractionation of isotopes during the various steps in the analysis.[9, 10] The first problem has been solved by a ratiometric technique of high precision employing a dual-collector mass spectrometer; each collector monitors the signal from one of the two isotopically distinct molecules, $^{13}CO_2$ or $^{12}CO_2$. Second, the issue of variable isotopic abundance in foods was resolved by studying the patients after an overnight fast and prohibiting the ingestion of drugs or food during the collection. Third, preservation of absolute isotopic integrity proved to be impossible, due to the partial trapping of expired CO_2 in NaOH. However, the fractionation curve was parabolic in shape and fractionation was almost constant in the range of yields obtained by bubbling expired air through alkaline solution in a 250 ml round-bottomed flask.

The conventional GC-^{14}C breath test is both the model and the standard for the ^{13}C breath test described here. The radioisotopic test is not without its inherent limitations, as pointed out by its developers. The test alone does not distinguish between small intestinal bacterial overgrowth vs. bile salt malabsorption.[5] Measurement of fecal isotope excretion may be of some usefulness in this distinction[5] when ^{14}C is employed but fecal isotope measurements are currently beyond practical feasibility with the stable isotope. Furthermore, as ^{14}C glycine is not stoichiometrically converted to $^{14}CO_2$, the degree of bile salt deconjugation is underestimated.[6] The test must therefore be considered semiquantitative[6] and less quantitative than direct intubation and analysis of aspirated fluids.[5] Despite these limitations, favorable conclusions regarding the clinical utility and varied applicability of the GC breath test in large series of patients have been reported.[13, 14]

An interesting and important finding in our study was the statistical identity of the response of the conjugated bile salt labeled with ^{13}C on the number one carbon of glycine and of the compound labeled with the stable isotope on both carbons of glycine. Our findings confirm the previous observation[5, 6] that hydrolysis of cholylglycine rather than oxidation of glycine to CO_2 is the rate-limiting and discriminating step in the GC breath test.

The correlation between the simultaneous GC-^{14}C and GC-^{13}C breath tests in the same patient is sufficiently strong and consistent to validate the stable isotope test. The reason for the observed lack of a 1 : 1 correspondence of calculated bile salt deconjugation is not fully understood, but the chief consequence of this isotopic behavior is to decrease

the sensitivity of the breath test in the lower ranges of deconjugation. In practice, 6% to 10% deconjugation (in terms of the GC-^{14}C response) could be readily detected with the 1,2-^{13}C compound given in a dose of 7.5 mg/kg. This sensitivity would also be obtainable with the use of the 1-^{13}C substrate in a dose of 15 mg/kg of body weight, but the latter dose is excessively large relative to expected bile salt pool and too irritating and unpalatable in aqueous solution to have been considered for trial in this study. Thus our glycocholate-1-^{13}C-glycine breath test is less sensitive at the low range in determining altered bile salt handling than the ^{14}C analogue but would be able to distinguish normal and abnormal situations in the majority of cases of gastrointestinal pathology that are associated with excessive bile salt deconjugation.

Experience in 13 subjects has shown a glycocholate-^{13}C-glycine breath test to be acceptable with minimal side effects. Most patients reported a pungent, bitter taste for the bile salt solution, and one patient experienced a diarrheal episode lasting for less than 24 hr following his test. Even including these experiences, we conclude that the ^{13}C breath test, unlike the analogous ^{14}C breath test, is without risk. At present, the cost of small-scale syntheses of the ^{13}C-labeled substrates is expensive, and the availability of mass spectrometric equipment for determination of ^{13}C abundances in clinical samples is limited. Nevertheless, the demonstration of the feasibility of obtaining equivalent diagnostic information without exposure of the patient to radioactivity may be expected to alter these circumstances. Initial use of a central facility to analyze samples collected at distant points will make the analytical measurements accessible to more clinicians, and visualization of the demand for the substrate will bring a reduction in the cost per patient. Finally, the applicability of this and other ^{13}C breath tests in groups such as infants, pregnant women, and women at risk of pregnancy will bring diagnostic benefits to these groups that are presently denied them because of the radiation hazard of ^{14}C-labeled compounds.

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