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STABLE ISOTOPES: ESSENTIAL TOOLS IN BIOLOGICAL AND MEDICAL RESEARCH

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The renaissance of interest in stable isotopes of hydrogen, carbon, nitrogen, oxygen and sulfur within the last ten years is based upon the development of new instrumentation, on the greater availability of enriched isotopes, and on an espousal of non-radioactive techniques for human studies on ethical grounds. In order to put the present use of stable isotopes into perspective, we should examine both its antecedents and its expectations, so that we can judge the benefits to be expected.

If one pursues the trail of papers on stable isotopes backwards in time, it stretches back 50 years to 1927, when Aston first described a new mass spectrometer with a resolution of 600. This instrument showed the presence of three sulfur isotopes at masses 32, 32 and 34 (Aston, 1927) and prompted him to espouse the whole number rule for elements, as opposed to the fractional masses obtained by chemical analysis. There followed within quick succession the detection of ^{13}C in optical spectra from graphite arcs (King and Birge, 1929), ^{18}O and ^{17}O in atmospheric solar spectra (Babcock, 1929), (Giauque and Johnston, 1929), ^{15}N in the absorption bands of NO (Naudé, 1931) and finally, the discovery of deuterium (Urey, Brickwedder and Murphy, 1932). This sequence of discovery was not accidental; it hinged on the abundance of each isotope and the available means of detection and thus went from 4.3% for ^{34}S to 0.015% for ^2H within a five year period.

On the other hand, the production of enriched fractions of the stable isotopes was achieved on the basis of the magnitude of the isotope effect each exerted in a physical or chemical process, and it was deuterium, the last isotope to be discovered that was first produced in highly enriched form in Urey's laboratory. According to Clarke (1948), the first toxicological studies on D_2O were

conducted within a year after the first published report of the existence of deuterium and tested the ability of goldfish, earthworms and protozoa to survive in 92% D₂O (Taylor et al, 1933). Urey was fully aware of the tracer potential of deuterium and through a grant from the Rockefeller Foundation was able to subsidize the production of heavy water and attract a young Ph.D., David Rittenberg to whom he gave the commission to find applications for the isotope. The heritage of that grant, in the collaboration of Schoenheimer and Rittenberg, is difficult to over-estimate, for it brought the concept of a dynamic equilibrium in metabolic processes to the then-static physiological chemistry of the day. Clarke's account of these fruitful years from 1933 through 1942 deserves periodic re-reading to keep in mind just how extensively the findings from the use of deuterium, and later, ¹³C and ¹⁵N defined the areas of modern biochemistry.

In September of 1947, a symposium was held at the University of Wisconsin entitled " The Use of Isotopes in Biology and Medicine". It marked the watershed in stable isotope useage, for in 1945, the first shipments of ¹⁴C had been made by the newly established Atomic Energy Commission. In this historic meeting, the two techniques for methods of preparation, synthetic procedures, instrumentation, and quantitation; one for stable isotopes and the other for radioactive isotopes were presented side by side. The sense of excitement at the potential of radioactive tracers, then newly introduced is keenly evident in the proceedings, and the literal explosion of radioactive tracer studies in the next decade completely overshadowed deuterium and ¹³C applications.

The reasons are quite clear: detection was simpler, required far less sample preparation, and as we all know, was soon fully automated. Whereas the availability of stable isotopes depends upon the magnitude of the isotope effect exerted by the difference in mass, and upon the starting abundance of the desired isotope, no such limitations exist for radioactive isotopes. Stable isotope production is ultimately limited by the thermodynamic considerations, whereas radioactive isotopes depend only on the flux of neutrons available, and the higher the flux, the higher the specific activity of the isotope produced. Introduction of liquid scintillation counting in the late 1950's should perhaps be regarded

as the great equalizer of tracer methodology, because this equipment made it possible for any research worker, regardless of previous experience, to enter the field.

All of this transpired more than 25 years ago, and in our present experience we are faced with the search for significance in the use of stable isotopes. Clearly the halcyon days of 1940, teeming with ground breaking opportunities in every experiment are gone, not likely to return. Nor are the majority of radioactive isotope users likely to foresake the ease and convenience of liquid scintillation counting for the headaches of gas chromatography-mass spectrometry that we all know and experience daily. It is true that when there are no alternatives, the slow, labor intensive mass spectrometric measurement of stable isotope ratios will go on at any cost. To appreciate this fully, one need only tot up the number of papers dealing with ^{15}N applications in agricultural science journals. Similarly, the absence of useable radioactive oxygen isotopes has created an exclusive demand for ^{18}O and ^{17}O that is note likely to be challenged. It is also true that both deuterium and ^{13}C are essential for the determination of fragmentation pathways, but, it should be emphasized, these are usually non-recurrent applications, just as the introduction of ^{13}C into an antibiotic for NMR studies is a unique event.

Where then are the areas of application that justify our convening in various spots around the globe at frequent intervals? It is clearly not simply the choice between using radioactive versus stable isotopes in human studies, as Dr. Hofmann will illustrate, because the use of 1-10 μCi of ^{14}C or 10-50 μCi of ^3H has not been banned in adults, nor is it likely to be, under the present risk/benefit ratio. We need to remind ourselves that if, in the attending physician's opinion, a procedure requiring the administration of radiation or radioactivity is clearly beeneficial to ~~the~~^y diagnosis or prognosis of the patient's condition, the procedure will be used. This includes the use of external emitters in scanning techniques in infants or women of child-bearing age, which involve higher doses of radiation that would be encoutered in most tracer applications.

On the other hand, ethical considerations require that the physician avoid any procedure that is not both harmless and non-invasive in normal patients. Without a doubt, these limitations are most stringently imposed when the studies involve pre-term or term infants, children and women in potential or actual child-bearing status. Since pregnancy is a transitory state in health care delivery, there have been few studies reported in which tracer techniques were required to evaluate the state of the pregnancy or of the conceptus, and where stable isotopes have actually been used. On the other hand, stable isotopically labeled drugs have been used by Dr. Marjorie Horning in studies of lactating mothers (Horning et al, 1973); kinetic measurements of bile acid metabolism have been conducted in infants (Watkins et al, 1973, Watkins et al, 1975); alanine and glucose fluxes have been determined in infants by Bier (Bier et al, 1976) and the pathway of deuterated aromatic amino acid metabolism has been mapped in infants by Curtius (Curtius, Völlmin and Baerlocher, 1973), to name but a few examples. It seems clear that there is a large body of information on adult metabolic processes waiting to be extrapolated to the child and infant. Extrapolation of this information will, in many cases, extend the diagnostic and therapeutic benefits of adult experience to the child, that are presently denied for lack of it, and pediatric applications of stable isotopes seem assured of considerable expansion.

It is in the area of pharmacology, however, that two types of applications appear to be particularly dependent upon the use of stable isotopes for recurrent measurements. (The use of stable isotopic labeling to provide ion pairs useful in drug metabolite identification developed by Hammar, Holmstedt and Ryhage (1968) is excluded in these considerations because of its non-repetitive application in the same molecule) The first of these is in the measurement of drug or metabolite concentration in plasma when the circulating level is at or below the microgram level and labeled forms are used as carriers and internal standards. The second is in the measurement of drug kinetics in plasma when these low levels are present, and when the total body pool is large. These two applications are best exemplified in our own experience in the study of methadone metabolism in humans, as conducted

at Rockefeller University by Dr. Mary Jeanne Kreek.

A penta-deuterium labeled methadone has been synthesized by Dr. Hachey according to the scheme shown in figure 1. This material, which can be provided as the racemate or as individual optical isomers is used either as the internal standard for quantitative measurements of plasma and urinary levels, or as the pharmacological maintenance dose in kinetic measurements. (Hachey, Kreek and Mattson, 1976). Similar studies have also been reported by Dr. Ånggård and his co-workers (Ånggård et al, 1976). Figures 2 and 3 show the quantitation of methadone in plasma and in urine, respectively, and illustrate the linearity of more than 3 orders of magnitude that can be achieved with this d_5 internal standard.

Methadone metabolism is unusual in that the plasma levels of the drug fluctuate within relatively narrow, but low, levels: typically 100-500 ng/ml, even following the daily oral dose of approximately 1 mg/kg. The excursions in plasma values on two successive days in a patient are shown in figure 4, which also compares the values obtained by gas chromatography (which employs ^{14}C methadone to monitor extraction from plasma) with those obtained by gas chromatography-chemical ionization-mass spectrometry. The levels determined by GC-CI-MS are consistently higher than those by GC alone, and when the results are plotted for correlation (figure 5), it appears that the GC-CI-MS technique "sees" more methadone than the GC technique. Since this discrepancy does not occur in urinary measurements, it appears to be a reflection of the effect of the plasma protein matrix and may be a source of additional information on the binding of this drug in plasma. Two possibilities have occurred to us, the first related to methodology, the second to sample history. In the first case, the ratio of internal standard to endogenous methadone is from one to five times as much d_5 as d_0 , whereas in the ^{14}C methadone, it is one tenth to one twentieth as much. If binding to the plasma proteins differs in adsorption coefficients, the smaller "spike" may not fully equilibrate prior to extraction. Preliminary studies to establish this have not been supportive of this possibility. The second possibility lies in the fact that the GC-CI-MS measurements were performed on the same plasma samples after two to three more cycles of freezing and thawing, and this may have denatured

protein sites and rendered more methadone accessible for exchange and extraction. Studies are also presently underway to investigate this possibility.

It is informative to calculate how much radioactivity is required to conduct pharmacokinetic studies in plasma when the circulating drug levels are low in comparison to the total body burden. Table 1 lists the assumptions that were used by Dr. Schoeller in calculating the radioactivity required for measurements having $\pm 5\%$ or $\pm 1\%$ counting accuracy. These values are 20 pCi and 457 pCi respectively. Depending upon the concentration of the drug in plasma, the amount of radioactivity required to produce these levels, when the body burden is 1 gm, range from 0.2 μ Ci to amounts that exceed the levels of specific activity found in carrier-free material. It can be seen for example, that even with counting accuracies of $\pm 5\%$ it is difficult to follow the plasma kinetics of a metabolite in the 10-100 ng/ml range without exceeding the limits of safety. On the other hand, it is feasible to administer the entire dose in a stable isotopically labeled form, either in a single or multiple dose without hazard. Since the physical quantity of labeled material is not a limiting factor, one can achieve labeling concentrations in the range where stable isotopic ratio determinations can be carried out on the quantities available in 1 ml of plasma. This permits the labeled drug to be followed for a much longer period of time. Figure 6 illustrates the isotopic abundance in plasma methadone in a maintenance patient following the substitution of a single dose of d_5 methadone for the regular daily dose. The existence of two pools is suggested by the computer fit to a double exponential function, one with a half life of 1.9 hours and a second with a half life of 31.4 hours. Note that isotopic ratio measurements could be carried out for more than a week before the sample size became limiting. In urine, where the concentration was higher, it was possible to follow the disappearance of label for more than ten days, as shown in figure 7. These measurements also suggested two kinetic components with half lives of 7.2 and 46.2 hours respectively.

A number of patients have been studied with the use of the d_5 methadone, and we are particularly interested in the drug-drug interaction opportunities that present themselves in methadone maintenance patients, such as concurrent alcohol

or barbiturate use, or therapy with rifampin (Kreek et al, 1976). From the standpoint of stable isotope useage, however, several aspects of this work deserve further comment as they bear upon the future of these techniques. First, even the relatively simple extraction procedures used in these studies and the short gas chromatographic retention times become limiting factors in the speed with which samples can be processed. When the total number of sqmples involved in plasma, urine and fecal kinetic measurements are considered, it is evident that these studies are labor-intensive, compared with radioactive studies in animals. Moreover, because the instrumentation cost is high, it is not feasible in most laboratories to run such studies in parallel on two or three instruments at the same time. This emphasizes the aspect of stable isotope applications that require GC-MS which must be kept uppermost: they should be low volume:high information gain problems where at all possible. The present effort required to measure isotope ratios in intact organic molecules means that problems involving large numbers of samples will have limited opportunities to be explored.

It was for these reasons that we began several years ago to look at stable isotope applications in which the end measurement of isotopic abundance could be simplified. In the area of clinical applications, it seemed that the then-existent ¹⁴C "breath tests" were beginning to show significant diagnostic potential and that the substitution of ¹³C labeled substrates would permit these diagnostic procedures to be used in children. The analytical requirement was then to determine the abundance of ¹³CO₂ in respiratory CO₂, and since all of the tests had in common the evolution of labeled CO₂, a single procedure could accomodate a diversity of applications. A major contribution to defining the analytical system requirements arose from the work of Dr. Schoeller and his associates (Schoeller et al, 1976) who were able to document the limiting precision of respiratory ¹³CO₂ measurements. Of the total variance in replicate measurements over a 6 hour period in fasting patients (0.518 per mil), 0.470 was due to metabolic variations in endogenous ¹³CO₂ abundance, even under fasting conditions (table 2) . This meant that the 95% confidence limit for a significant change in isotopic abundance

of the respiratory CO_2 was 1.4 per mil or 0.14‰. The significance of this finding is two-fold: it defines the limiting instrumental sensitivity required (far less than originally suspected, and much more easily achieved) and it defines the minimum substrate oxidation rate that can be anticipated to be detectable against the background of endogenous $^{13}\text{CO}_2$ and its variation. This rate must be in excess of 0.14 $\mu\text{M/kg/hr}$, and should preferably be 5-20 times as large. This enables us to rule out, on the basis of radioactive measurements, those substrates whose oxidation would be undetectable if they were labeled with ^{13}C instead of ^{14}C . For example, aminopyrine produces a significant increase in $^{13}\text{CO}_2$ abundance when the N-dimethyl ^{13}C form is administered, but the oxidation rates of ^{13}C methyl labeled valium or methadone would not be detectable with physiological doses.

A substantial component of the cost of stable isotopic ratio measurements is the labor involved in processing samples. It seemed to us that the automation of $^{13}\text{CO}_2$ isotope ratio measurements would effect a considerable economy, and we have been pursuing this objective. A key requirement is the ability to collect, ship and store individual samples of respiratory CO_2 in a manner that is simple, inexpensive and, hopefully, without effect on the isotope content. This problem has been solved by using evacuated 50 ml containers, sealed with rubber septa, and available commercially as Vacutainers. Figure 8 illustrates the stability of isotope abundance measurements on Vacutainer samples over a 7 week period and the comparison of these values to those obtained on CO_2 trapped in NaOH and released by acidification. (Schoeller and Klein, 1976). An additional bonus is that the isotopic depletion that occurs when CO_2 is trapped in a dynamic flow system in NaOH is absent when Vacutainers are used and the absolute value of the $^{13}\text{C}/^{12}\text{C}$ abundance ratio is preserved.

At the present time, we are constructing a prototype system for automatic $^{13}\text{CO}_2$ measurements, using Vacutainers as sample containers. The flow chart for the steps in the analysis are shown in table 3, and the mechanical steps therein are being programmed for control by a dedicated microprocessor that also serves to collect the ion current ratio data. The ultimate objective, of course, is to be

able to load a rack of fifty samples into the system at the end of the clinical working day and have answers printed out by the next morning. Furthermore, it is our conjecture that if discrete samples can be analysed in this manner, combustion processes for total label recovery, or selective degradation steps can also be programmed for automatic analysis. Such a capability should increase the opportunities for the clinical applications of ^{13}C labeled compounds by eliminating the analytical bottlenecks that presently exist.

As we begin the second fifty years of stable isotope applications, there are still many of the original limitations of cost and difficulty present in the beginning. Nevertheless, we appear to be moving into a new era of human studies in which the answers we seek cannot be obtained in any other way, and once more, stable isotopes have become essential tools in biological and medical research.

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Table 1. Calculation of radioactive tracer dose required for kinetic measurements in plasma

Assumptions

Pool size: 1 gm	Molecular weight 400
Counting efficiency: 100%	Countin time 10 minutes
Background rate: 10 dpm	Labels/molecule: 1

Intermediate data

Counting accuracy: $\pm 1\%$ requires 1005 dpm or 457 pCi
 $\pm 5\%$ requires 44 dpm or 20 pCi

For 1 ml plasma samples, required dose administered is:

<u>Concentration</u>	<u>1% accuracy</u>	<u>5% accuracy</u>
1 mg/ml	0.46 μ Ci	0.2 μ Ci
1 μ g/ml	457 μ Ci	20 μ Ci
1 ng/ml	Exceeds carrier-free specific activity	20,000 μ Ci

Table 2. Variance introduced in each step of $^{13}\text{CO}_2$ analysis

<u>Source</u>	<u>Variance</u> <u>σ^2</u> <u>/100</u>	<u>Number of analyses</u>
Total variance	0.518	39
Ratio determination	0.002	10
CO_2 release	0.017	9
CO_2 collection	0.029	14
Residual (due to patient)	0.470	

Table 3. Automated breath $^{13}\text{CO}_2$ analyser

Puncture Vacutainer^r septum and withdraw an aliquot

Pass gases through a dry-ice trap to remove water

Collect the CO_2 in a liquid nitrogen trap

Transfer the CO_2 to the mass spectrometer inlet

Adjust the volume to produce a desired pressure

Measure the ratio of $^{13}\text{CO}_2$ to $^{12}\text{CO}_2$ in the sample

Measure the ratio of $^{13}\text{CO}_2$ to $^{12}\text{CO}_2$ in a reference

Calculate and printout the $^{13}\text{CO}_2$ enrichment

Advance to the next Vacutainer^r and begin again

Figure Legends

Figure 1. Synthesis of d_5 labeled methadone

Figure 2. Plasma methadone inverse isotope dilution curve

Figure 3. Urinary methadone inverse isotope dilution curve

Figure 4. Plasma methadone levels in a patient over a 36 hr period measured by gas chromatography and gas chromatography mass spectrometry.

Figure 5. Correlation of plasma methadone values obtained by gas chromatography with those obtained by gas chromatography mass spectrometry.

Figure 6. Plasma disappearance of methadone following a single 100 mg oral dose of methadone d_5 .

Figure 7. Urinary excretion of methadone following a single oral 100 mg dose of methadone- d_5 .

Figure 8. Stability of isotopic abundance values of respiratory CO_2 stored in Vacutainers, or trapped as $NaHCO_3$ in NaOH

I

II a = (±)-

b = (+)-

c = (-)-

III a-c

Plasma methadone inverse isotope
dilution curve constructed using
540 ng internal standard.

Urinary methadone inverse isotope
dilution curve constructed using
540ng internal standard.

Plasma Disappearance of Methadone
Following a 100 mg Oral Dose
of Methadone-D5.

Urinary Excretion of Methadone Following a 100 mg Oral Dose of Methadone-D5.