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Stable Isotope Tracers in the Life Sciences and Medicine

Increased supplies and improved detection methods of ^{13}C and other stable isotopes permit their wider use as tracers.

N. A. Matwiyoff and D. G. Ott

The separated stable isotopes of carbon, oxygen, and nitrogen have been available in limited quantities for many years. These isotopes, some properties of which are listed in Table 1, are nonradioactive, naturally occurring forms of the elements. Since they are important components of all organic compounds which occur in nature, the isotopes have a great potential utility in tracer and structural studies in the life sciences and medicine. Indeed, the use of ^{13}C and ^{15}N in physical and biological sciences predates the widespread use of the radioactive isotope ^{14}C (*1*). Despite their great promise, little use has been made of the stable isotopes of these elements, for two main reasons. First, they were expensive because they were prepared and used in very limited amounts. Second, instruments necessary for the detection and assay of stable isotopes were not widely available and were difficult to maintain.

It now appears that not only can the difficulties associated with the methods of detection of stable isotopes be resolved, but also the supplies of such isotopes and the technology for concentrating them have improved dramatically within the last several years. In this article we review the isotopes listed in Table 1, focusing in particular on ^{13}C because recent developments have been the most substantial and dramatic for this isotope. We omit much discussion of deuterium because it has been reviewed recently elsewhere (*2*).

Methods for detecting stable isotopes used as tracers are not as sensitive as those for detecting radioactive tracers. This is because of the high natural abundance of stable isotopes. For example, analytical methods are now available for detecting 0.1 percent changes in the ratio of ^{13}C to ^{12}C and, consequently, studies can be accomplished with tracer molecules simply labeled with ^{13}C in which the tracer is diluted to 1 part in 100,000. Even though such a dilution is not as great as that attainable in radioactive tracer experiments, useful studies can be accomplished provided that there are plentiful supplies of inexpensive stable isotopes. Much more sensitive tracer experiments are possible if molecules are multiply labeled with stable isotopes —the calculated natural abundance of the carbon dioxide molecule, $^{13}C^{18}O_2$, is only 4.4×10^{-6} percent and studies of carbon dioxide transport are possible in principle with this multiply labeled molecule in which the label undergoes a dilution as high as 1 part in 10^9 with natural carbon dioxide, $^{12}C^{16}O_2$.

These limitations on the minimum changes detectable in stable isotope ratios, which are only in part analytical limitations, suggest that stable isotopes will complement rather than replace radioisotopes as tracers. What then are their special characteristics that forecast an important role for them in the biological sciences and medicine? First, they are not radioactive. With the increasing concern about exposure to even small amounts of radioactivity, the use of stable isotopes is clearly advantageous in environmental studies and clinical medicine. Even in laboratory research, the avoidance of radioactive tracers can be highly desirable because of the possibility of radiochemical breakdown during the stockpiling of ^{14}C-labeled compounds, for example. In addition, although the radioactive nuclides of carbon and sulfur provide great sensitivity in tracing experiments, there are no long-lived radioactive isotopes of nitrogen or oxygen.

Second, in the use of isotopes with a nonzero nuclear spin, detection by nuclear magnetic resonance (NMR) spectroscopy usually allows one to distinguish, for example, not only the ^{13}C or ^{15}N content but also the exact location of the isotope in a molecule or complex mixture. Under favorable conditions the location in the molecule of a stable isotope label can also be specified from a comparison of the mass spectrograms of the labeled and unlabeled molecules. In contrast, the localization of a radioactive tracer in a given molecule or mixture requires tedious chemical degradations and product separations followed by radioassay. Even so, the presence of radioactive tracers usually can be detected easily because very sensitive methods are available; consequently, radioactive tracer methods are most efficient for the rapid assay of a label which is distributed among a large number of complex fractions. In that regard, the use of stable and radioactive isotopes are complementary—once the labeled fraction has been identified by radioassay, the precise chemical location of the tracer can be studied with stable isotopes.

We will now describe some important recent developments in the separation, chemical conversion, and quantitative detection of stable isotopes, referring especially to ^{13}C, and will then discuss the potential uses of these isotopes in the life sciences and medicine.

Dr. Matwiyoff is alternate group leader of group CNC-4 and chairman of the Steering Committee on Stable Isotopes. Dr. Ott is group leader of group H-11 at the Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico 87544.

Isotope Separation

Isotopic substitution in a molecule is accompanied by many changes in the chemical and physical properties of the molecule. Such changes are small, especially for the heavier elements, but some of them can be exploited in isotope separation schemes. In the equilibrium between the vapor and liquid forms of carbon monoxide and nitric oxide, the effects of the isotopes on the vapor pressure partitions the heavy isotopes preferentially into the liquid phase as summarized in the equilibrium constants, 1.027 and 1.008, for processes shown in Eqs. 1 and 2, respectively. Because the equilibrium constants are small,

$$\underset{\text{liq.}}{^{14}NO} + \underset{\text{gas}}{^{15}NO} \rightleftarrows \underset{\text{liq.}}{^{15}NO} + \underset{\text{gas}}{^{14}NO} \quad (1)$$

$$\underset{\text{liq.}}{^{12}CO} + \underset{\text{gas}}{^{13}CO} \rightleftarrows \underset{\text{liq.}}{^{13}CO} + \underset{\text{gas}}{^{12}CO} \quad (2)$$

the separation obtained in a single stage of distillation is slight and long distillation columns having a large number of theoretical plates are required to effect high enrichments of the low abundance isotopes and depletion of these isotopes in the fraction containing the major isotope. The relatively large effects of isotopes on the vapor pressure of NO together with the facile isotope exchange equilibrium (Eq. 3) which occurs in NO

$$^{14}N^{18}O + {}^{14}N^{17}O + {}^{15}N^{16}O \rightleftarrows {}^{15}N^{17,18}O_2 + {}^{14}N_2{}^{16}O \quad (3)$$

allows the separation of oxygen isotopes during the distillation of NO.

At the Los Alamos Scientific Laboratory (LASL), the isotope separation facility produces ^{13}C (90 percent) in two columns at a rate of 6 kilograms per year and the average production of ^{12}C (less than 50 parts per million of ^{13}C) in one column has been 12 kilograms per year. Smaller amounts of nitrogen and oxygen isotopes have been produced in a variety of column systems (*3*). An expanded facility for the separation of stable isotopes is now under construction at LASL and is expected to increase greatly the available amounts of the various isotopes (*4, 5*).

Isotopes can also be separated by chemical exchange reactions. For example, the equilibrium constants for processes shown in Eqs. 4 and 5

$$\underset{\text{gas}}{^{15}NH_3} + \underset{\text{aq.}}{^{14}NH_4{}^+} \rightleftarrows \underset{\text{gas}}{^{14}NH_3} + \underset{\text{aq.}}{^{15}NH_4{}^+} \quad (4)$$

$$\underset{\text{aq.}}{^{12}CO_3{}^{2-}} + \underset{\text{gas}}{^{13}CO_2} \rightleftarrows \underset{\text{aq.}}{^{13}CO_3{}^{2-}} + \underset{\text{gas}}{^{12}CO_2} \quad (5)$$

Table 1. Some characteristics of nonradioactive isotopes.

Isotope	Natural abundance (%)	Nuclear spin
^{12}C	98.9	
^{13}C	1.1	1/2
^{14}N	99.6	1
^{15}N	0.4	1/2
^{16}O	99.8	
^{17}O	0.04	5/2
^{18}O	0.2	

are 1.034 and 1.017, respectively (*6*), showing that the heavy isotope accumulates preferentially in the chemical form in which it is bound most strongly: $NH_4{}^+$ and $CO_3{}^{2-}$, respectively. This small but significant isotope effect is due to the mass effect on vibrational frequencies and zero point energies (*7*). The processes summarized in Eqs. 4 and 5 have been used to concentrate ^{15}N and ^{13}C by a countercurrent process in which the gas (NH_3 or CO_2) and aqueous salt (NH_4NO_3 or Na_2CO_3) solutions were passed through long columns, the heavy isotope accumulating in the salt solution and the light one in the gas stream (*8*). Other chemical processes have been developed to concentrate significant amounts of carbon, oxygen, and nitrogen.

Labeled Compounds

A major consideration in the design of tracer experiments is the availability of sufficient quantities of labeled material in the required chemical forms. The situation is reminiscent of that for ^{14}C and ^{3}H before significant quantities of a variety of labeled compounds became available commercially. It is reasonable to expect that the number of currently available compounds labeled with stable isotopes will soon be significantly increased. Lower costs will accompany increased demands that will, in turn, stimulate higher production levels (*5, 9*). Many different combinations of labeling are required—some compounds must be uniformly labeled, some specifically labeled in one or more particular molecular positions, and some multiply labeled with more than one isotope, both uniformly and specifically. For some uses, high isotopic enrichment is required; for others, low enrichment is necessary or sufficient. The quantities of materials needed can vary from milligrams to kilograms.

In the syntheses of isotopically labeled compounds, standard methods of organic and biochemistry can be used but, because the economics of the processes and the availability of starting materials must be considered, different approaches are often required. In this regard there are many similarities between the problems associated with ^{14}C syntheses and those with ^{13}C, but many of the schemes developed for the radioactive isotopes are usually not applicable to ^{13}C, largely because of the difference in scale of the preparations. For example, although the ^{14}C-labeled acetic acids can be readily prepared by way of the Grignard reaction, through acetonitrile, or from acetylene, preparation on a larger scale with ^{13}C is much more conveniently and efficiently based on catalytic methods with the most readily available starting materials (*10, 11*):

$$CH_3OH + CO \xrightarrow[\text{HI, } 175^\circ C]{RhCl_3} CH_3COOH \quad (6)$$

The procedure can be used to prepare all four of the carbon isotope isomers of acetic acid, as well as to prepare compounds labeled with ^{2}H, ^{17}O, or ^{18}O. The isotopic methanols are also conveniently produced by catalysis (*10, 12*):

$$CO \text{ (or } CO_2) \xrightarrow[\text{Cu-Zn-Cr}]{H_2} CH_3OH \ (+ H_2O) \quad (7)$$

That efficient production methods must be available for the simple organic compounds, for example, methanol, acetic acid, cyanide, and methyl iodide, before the more complex materials can be produced is illustrated in Fig. 1. The selection of a particular synthetic procedure (from among perhaps many possibilities) depends in a large part on previously established capabilities, techniques, and a ready availability of starting materials.

Biosynthesis is the preferred method for the preparation of uniformly labeled complex natural products as well as for certain specifically labeled materials; for example, hemoglobin labeled with [2-^{13}C]histidine is obtained from mice fed a diet devoid of natural histidine but with a [2-^{13}C]histidine additive (*10*). As encountered in organic synthesis, the efficacious biosynthetic methods perfected for radioactive isotope labeling frequently are not applicable on the scale needed for stable isotope synthesis. The scaling up of a procedure in which tens of grams of product must

be isolated and purified by chromatography may not be practical or even feasible. In stable isotope labeling through biosynthesis one must find a system that not only produces the desired product, but one that also affords the isolation of the product in a reasonable manner. Simple carbohydrates labeled with ^{14}C have been obtained for many years by means of photosynthesis from isotopic carbon dioxide by excised leaves of higher plants such as tobacco or Swiss chard. These procedures have been scaled up several thousandfold for preparations of the ^{13}C analogs, kilogram amounts of leaves being used together with multimolar quantities of CO_2 highly enriched with ^{13}C; endogenous (normal abundance) materials are depleted essentially to zero and no carriers are used—but these procedures have required considerable amounts of research and development (*13*). Isotopically labeled carbohydrates, amino acids, nucleosides, and lipids will probably be produced on a much larger scale by using large fermentors for the culture of microorganisms such as algae or photosynthetic bacteria.

The hydroponic growth of plants in an atmosphere of CO_2 is another approach to the synthesis of labeled compounds. For example, potatoes have been grown in an atmosphere containing 30 percent $^{13}CO_2$ and the starch has been isolated (*14*). For complex organic compounds produced by plants this will surely be a method of choice, but for the common intermediates of metabolism, such as sugars and amino acids, algae and excised leaf systems appear simpler at present.

Because the demand for materials labeled with stable isotopes will probably increase considerably in the near future, methods are now being developed which are capable of producing them on a scale resembling that on which deuterium labeled compounds are produced.

Analytical Techniques

A number of analytical methods are available for the determination of the stable isotopes in organic compounds. Those most widely used depend on mass effects (rotation, vibrational, and mass spectrometry) or the presence of a nuclear magnetic moment (NMR spectroscopy). The most common and convenient techniques available for isotopic analysis are listed in Table 2. With a few exceptions, the infrared (*15*) and optical emission (*16*) methods are restricted to simple molecules or gases which can be derived from the labeled ones by combustion (to CO_2) or oxidation (to N_2). Although the methods require simple, inexpensive instrumentation they cannot, in general, provide direct information about the location of the isotope in the molecule. In contrast, the NMR and mass spec-

Table 2. Some techniques used for isotope analysis.

Spectroscopic technique	Minimum sample size	Sample form
Infrared	Milligram–microgram	Simple gases (CO_2, NO)
Optical emission	Microgram	Simple gases (N_2)
Mass	Milligrams–picograms	Simple gases to complex mixtures
Nuclear magnetic resonance	Milligrams–micrograms	Simple molecules to complex mixtures

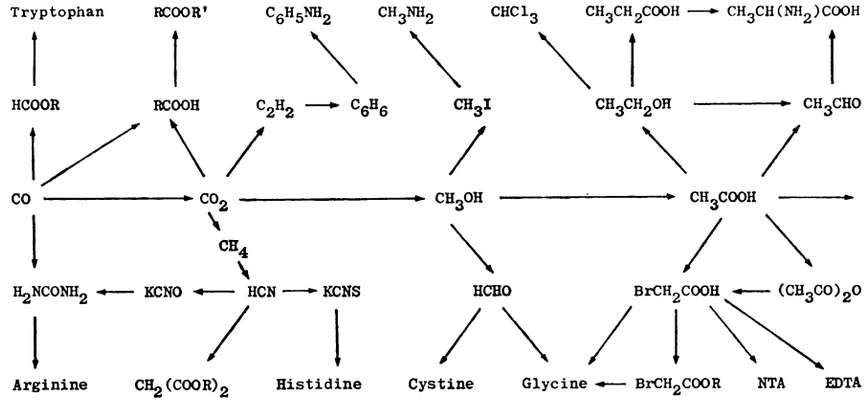


Fig. 1. The centralized isotope synthesis facility at Los Alamos allows an impressive array of complex labeled compounds to be synthesized from the simple intermediates: CO; CO_2; CH_3OH; and CH_3COOH.

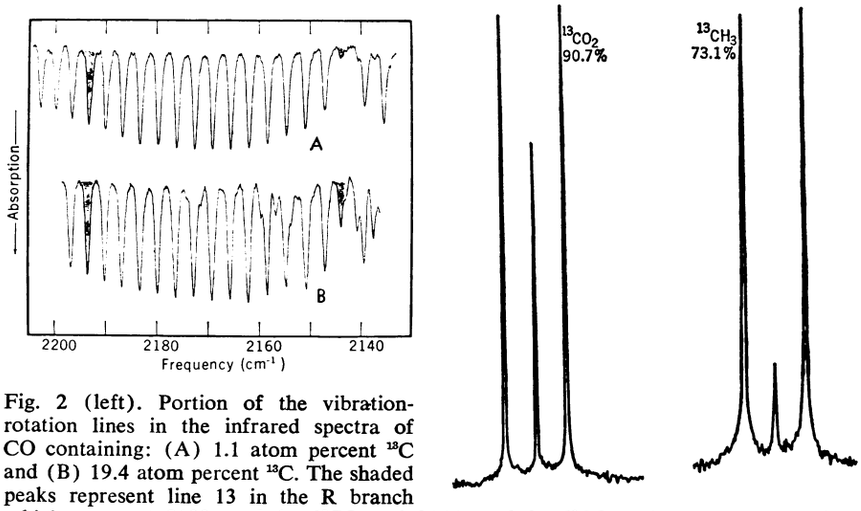


Fig. 2 (left). Portion of the vibration-rotation lines in the infrared spectra of CO containing: (A) 1.1 atom percent ^{13}C and (B) 19.4 atom percent ^{13}C. The shaded peaks represent line 13 in the R branch which occur at 2193 cm^{-1} for ^{12}CO and 2144 cm^{-1} for ^{13}CO and are sufficiently free from interference to serve for the determination of the $^{13}C/^{12}C$ ratio (*15*). Fig. 3 (right). The ^{13}C NMR spectra with noise-modulated proton decoupling for [1,2-^{13}C]acetic acid enriched to 73 percent ^{13}C at C-2 and 91 percent ^{13}C at C-1. The central line for each set of resonances arises from molecules containing ^{13}C adjacent to ^{12}C, whereas the flanking doublets arise from ^{13}C-^{13}C nuclear spin-spin splitting in molecules containing adjacent ^{13}C atoms. The intensity distribution within the doublet and singlet provide a quantitative measure of the concentration of ^{13}C at the adjacent position (*9*). The integrated intensity of each set of resonances is also proportional to the ^{13}C concentration at that position. The two sets of resonances were recorded at different instrument gain settings.

trometric methods of isotope analysis require more complex and expensive instrumentation but offer the advantage that the location of the label in a molecule can usually be determined. It should be noted that ^{17}O and ^{14}N have quadrupole moments and their nuclear resonances are frequently so broad that analysis by NMR spectroscopy is an insensitive procedure. Typical spectra obtained by some of the methods outlined are shown in Figs. 2 to 4.

Other analytical techniques, such as electron paramagnetic resonance (*17*) and Raman spectroscopy (*18*), have been used for the detection of stable isotopes and, although not in widespread use, these techniques offer much promise. Of particular interest is proton reaction analysis (*19*) which has been used to determine ^{12}C and ^{13}C isotopic abundances and the ratios of nitrogen to carbon in biological samples including human tissues. The technique, which involves the counting of gamma (γ) rays from the reactions ^{12}C (p,γ) ^{13}N and ^{13}C (p,γ) ^{14}N, offers the advantage of speed of analysis and the potential for accomplishing three-dimensional isotopic assays in complex specimens.

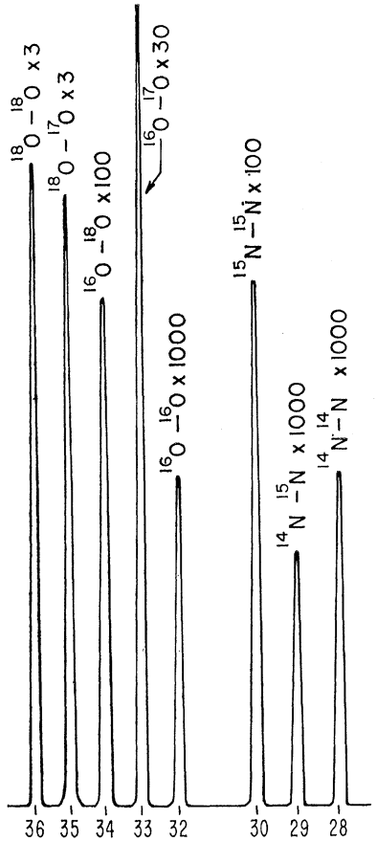


Fig. 4. Mass spectrum of an equimolar mixture of nitrogen and oxygen containing: 28 atom percent ^{15}N; 3.3 atom percent ^{17}O; and 6.9 atom percent ^{18}O. [Courtesy Vandervoort and Gomez (*61*)]

Improved Instrumentation Adds a New Dimension to Tracer Experiments

There is a substantial volume of literature on the applications of ^{15}N and ^{13}C tracers in the life sciences. Indeed, a recent bibliography (*20*) for ^{15}N lists no fewer than 1000 publications which appeared in the period 1935 to 1969. These applications generally depended on the spectrometric assay of excess ^{15}N or ^{13}C in a sample of N_2 or CO_2 obtained by careful separation and degradation of the molecule of interest. For example, in an early classic study of the use of stable isotopes, Shemin and Rittenberg (*21*) showed that glycine is a nitrogen precursor of the protoporphyrin of hemoglobin and used the result in the determination of the life-span of the red blood cell. [^{15}N]-Glycine was fed to rats and human beings and the incorporation of excess ^{15}N into the protoporphyrin was determined at intervals. The analysis was accomplished by mass spectrometry of N_2 derived from the separated and degraded porphyrin. Similarly, it has been shown (*22*) in a study of nucleated red cells of ducks fed [2-^{14}C]glycine that the α-pyrrole and methine bridge carbon atoms (indicated by solid circles in Fig. 5) of protoporphyrin-IX are derived from the α-carbon of glycine. In the same study, the location of the ^{14}C label was determined after a stepwise degradation of the porphyrin. A recent study of the incorporation of ^{13}C-labeled glycine into bacterial coproporphyrin-III (*23*) illustrates the power of NMR spectroscopy. Because the ^{13}C resonances are extremely sensitive to the location of the atom in a molecule, the site of incorporation of ^{13}C can usually be identified readily without the time-consuming degradations required in ^{14}C tracer methods.

Figure 6 shows the ^{13}C NMR spectrum with noise-modulated proton decoupling of an aqueous (5 m*M*) solution of coproporphyrin-III isolated from a culture of the purple bacterium, *Rhodopseudomonas spheroides*, grown under conditions in which coproporphyrin-III synthesis is induced on a medium containing glycine, 93 percent of which is labeled in the α-position with ^{13}C. The set of high field signals (at the right in Fig. 6) which, in the proton coupled spectrum appear as doublets, can be assigned to the methine carbon atoms and the low field set (on the left in Fig. 6) to the pyrrole α-carbon atoms which are only remotely coupled to protons. The resonances of the other ^{13}C atoms which are present at natural abundance ($\sim$ .05 m*M*) were not found in the spectrum. Because the tetrapyrrole synthesis proceeds via the intermediate formation of δ-aminolevulinic acid, porphobilinogen, and dipyrrylmethanes (*24*), these results also indicate the absence of much unlabeled glycine in the system.

It is probable that the technique of tracing ^{13}C by means of ^{13}C NMR spectroscopy will be widely used in studies of biological systems in the very near future. A number of such studies have been completed already. Acetic acid labeled with ^{13}C in the carboxyl and methyl groups and traced by NMR spectroscopy has been used to confirm the polyacetate origin of the microbial metabolite radicinin (*25*) and to define the biosynthetic origin of certain of the carbon atoms of sterigmatocystin (*26*) and the antibiotic cephalosporin (*27*). NMR techniques also have shown that the methyl group on the carbon 1 of ring A of vitamin B_{12} does not originate from the δ carbon of δ-aminolevulinic acid (*28*). Under favorable conditions the presence of ^{13}C attached to a hydrogen atom in a molecule can be determined indirectly by means of proton magnetic resonance spectroscopy; this technique has been used to elucidate the origin of some carbon atoms in a number of microbial metabolites (*29*).

Because the nuclear relaxation rates

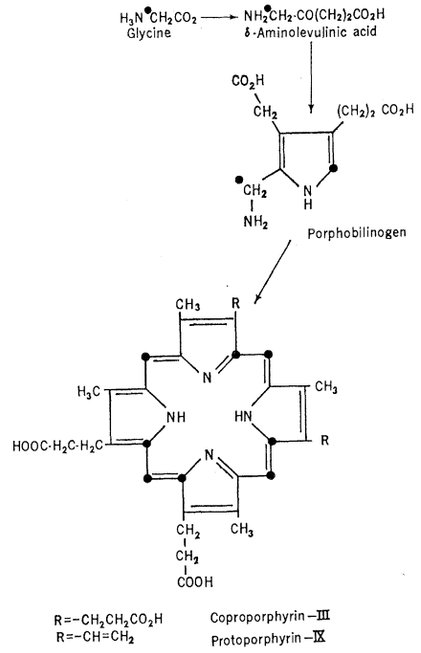


Fig. 5. Structure of protoporphyrin-IX, coproporphyrin-III, and the biosynthetic route to porphyrins. Solid circles indicate the location of the α-pyrrole and methine bridge carbon atoms.

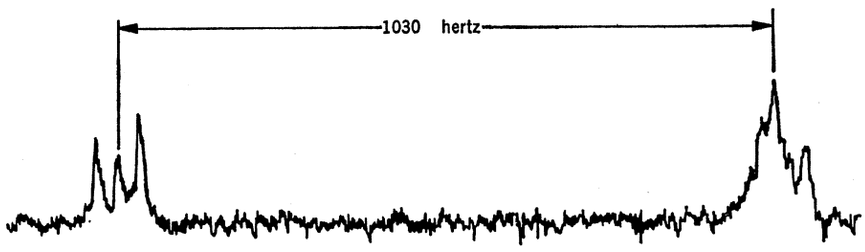


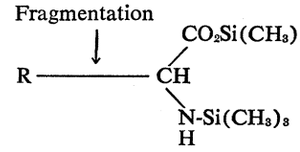
Fig. 6. ^{13}C spectrum with noise-modulated proton decoupling of coproporphyrin-III. The set of low field resonances of α-pyrrole consist of a doublet flanking a singlet, the former arising from those molecules containing ^{13}C in the adjacent methine sites (see Fig. 3). The ratio of the intensities of the doublet to the singlet shows that about 80 percent of the adjacent methine sites are occupied by ^{13}C. Unlike the pyrrole α-carbon atoms which have identical ^{13}C shifts, there are small chemical shift differences among the methine carbon atoms which resonate at high field. In an expanded version of the spectrum, a triplet (T), doublet (D), and singlet (S) resonance are discernible, consistent with the labeling pattern expected (as indicated by the solid circles in Fig. 5) and the high degree of ^{13}C incorporation: ●—●—● (T); —●—● (D); —●— (S).

of ^{13}C in most molecules are relatively slow (*30*), ^{13}C resonances are frequently narrow even in highly viscous systems and condensed phases. This is likely to be the case for ^{15}N resonances. This fortunate circumstance, together with the wide range of ^{13}C and ^{15}N chemical shifts, allows high resolution NMR studies of many solid and multiphase systems. Duch and Grant (*31*) have demonstrated that high resolution ^{13}C NMR spectra can be obtained from solid polymer samples and Sternlicht and co-workers (*32*), have obtained high resolution ^{13}C NMR spectra of amino acids bound to rigid cation exchange resins. The ability to record high resolution ^{13}C NMR spectra under these conditions suggests that ^{13}C NMR spectroscopy in conjunction with ^{13}C labeling could have a number of interesting applications ranging from the study of transport phenomena in multiphase systems to the elucidation of metabolic pathways in vivo. For example, distinct ^{13}C resonances of CO_2 bound in the form of dissolved CO_2, HCO_3^- ion, and carbaminohemoglobin (CO_2 bound to the terminal valine residues of hemoglobin, $O_2C-N\sim$) have been distinguished in the ^{13}C NMR spectra of human whole blood treated with $^{13}CO_2$ (*33*). High resolution ^{13}C NMR spectra have also been reported for ^{13}C enriched yeast cell suspensions, and the anaerobic metabolism of [1-^{13}C]glucose to [2-^{13}C]ethanol by yeast has been followed by ^{13}C NMR spectroscopy of the suspension of living cells (*34*). Although developments in ^{15}N NMR spectroscopy have not been as rapid, activity in this promising area has been increasing in recent years (*35*).

Substantial progress has been made in mass spectrometry instrumentation (*36*) and in the correlations of mass spectra with molecular structures (*37*), and it is now possible to measure directly the extent of incorporation, and the location, of a stable isotope label in a molecule. Frequently, the analysis of complex mixtures can be accomplished without chemical separation of the individual components. Thus, in 1961, the percent excess of ^{15}N present in 11 of the common amino acids enriched with different amounts of ^{15}N was determined by mass spectrometry of the esterified mixture of the amino acids (*38*). When ionized by an electron beam of low energy, each of the acids exhibits an intense peak at the decarboxylated fragment $(RCHNH_2)^+$ which occurs one mass unit higher than normal if the fragment contains ^{15}N. Since the R radicals present in all the common amino acids except leucine and isoleucine differ by more than one mass unit, each pair of peaks ($RCH^{14}NH_2^+$; $RCH^{15}NH_2^+$) represents a specific amino acid whose ^{15}N content can be calculated from the relative intensities of the peaks.

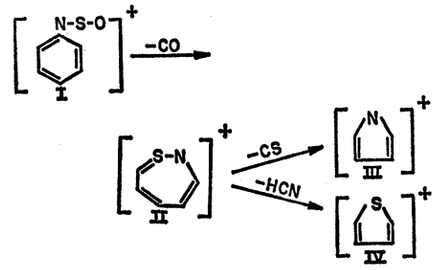
Some interference was encountered with other fragment ions of the heavy amino acids which coincide with the $RCHNH_2^+$ peaks of glycine, alanine, and proline (*38*). However, such interferences could probably be eliminated, or corrections made for them, by using modern techniques, such as the combination gas chromatography–mass spectrometer–on-line computer. Indeed, it appears that this technique can be used for the direct determination of amino acid sequences in the mixtures of peptides resulting from the degradation of proteins (*39*) and for determining the sequences of sugars (*40*).

Because extensive fragmentation can occur in a mass spectrometer, frequently in predictable ways, one can assay not only the isotopic content of a molecule but also the specific location of the isotopic label. For example, the mass spectra (*41*) of ^{13}C-labeled amino acids (12 to 15 percent) isolated from algae grown in an atmosphere of CO_2 (containing 15 percent ^{13}C) suggests that there is a small isotopic preference for ^{12}C in the biosynthesis of the amino acid side chain groups. The isotopic analysis was possible because the following fragmentation reaction of the trimethylsilyl derivatives of the amino acids occurred in the mass spectrometer:



These observations are consistent with the results of an earlier study (*42*) of the fixation of natural carbon dioxide by photosynthetic organisms in which it was found that serine, threonine, and glycine were somewhat more enriched in ^{13}C than leucine, isoleucine, and aromatic amino acids.

Conversely, one of the most powerful tools in the elucidation of molecular fragmentation patterns has been the stable isotope label (*43*). A simple example of the technique is the elucidation of the mechanism of loss of CO from the molecular ion of thionylaniline (*44*). The major fragmentation pathway of thionylaniline is



The loss of CO involves a major reorganization of the molecular ion. Which carbon atom is lost? The mass spectrum of [1-^{13}C]thionylaniline demonstrated that C-1 was not eliminated as CO, for the fragment ions I, II, and III all retained the ^{13}C label. With the aid of isotope labeling, the correlation of fragmentation patterns with molecular structure should become increasingly refined and the demonstrated power of mass spectrometry in structure determination (including the localization of functional groups and the specification of stereochemistry) will be extended greatly (*45*).

Heavy Macromolecules

Although we emphasize the use of stable isotopes as tracers, it is appropriate to point out here that plentiful supplies of stable isotopes will allow novel studies to be accomplished with multiply labeled macromolecules. A prototype is the classic experiment of Meselson and Stahl (*46*) who were able to demonstrate that the DNA of *Escherichia coli* is semiconservatively replicated. By culturing *E. coli* in a medium containing first $^{15}NH_4Cl$, then $^{14}NH_4Cl$, as the sole source of nitrogen and then separating "heavy" from "light" DNA by ultracentrifugation in a density gradient, they were able to show that after a single replication of DNA, the product molecules contained one strand of the original heavy material and one newly synthesized strand of light material. The use of stable isotopes and isopycnic techniques to effect the separation of newly synthesized macromolecules should find wide application in molecular biology and immunology (*46, 47*).

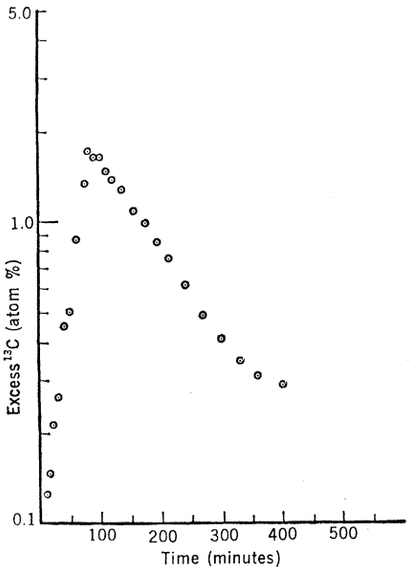
Potential Medical Applications of Stable Isotopes

The use of radioactive isotopes for medical, physiological, and pharmacological investigations in human beings has a long history. Although many clinical tests have been developed with radioactive isotopes, their use in reasonably healthy subjects, pregnant women, and children will probably remain limited because of the radioactivity. That stable isotopes will find broad application in studies of human beings, particularly in investigations of metabolic disorders, drug metabolism, and nutritional disorders and deficiencies, is suggested by the results of recent experiments.

One manifestation of a metabolic disorder is an acceleration or diminution in the rate of oxidation of a specific substrate to CO_2 by a diseased subject compared to a normal subject. As shown in Fig. 7, the rate of oxidation of a ^{13}C-labeled substrate can be followed indirectly by the determination of the rate of appearance of excess ^{13}C in the CO_2 exhaled in the breath (*48*). There is a large number of metabolic abnormalities that might be tested in this manner, two examples being lactase deficiency that might be tested with ^{13}C-labeled lactose, and hyperglycinemia that might be tested with ^{13}C-labeled glycine. The attractive features of the breath CO_2 test are simplicity and adaptability to mass screening of the population: the subject orally ingests the ^{13}C-labeled substrate, breathes into an evacuated bulb at intervals, and the excess ^{13}C in the breath CO_2 can be determined with inexpensive infrared or mass spectrometers. The utility of such tests in studying metabolic disorders has been demonstrated with ^{14}C. For example, is has been shown that patients with severe bile acid malabsorption excrete $^{14}CO_2$ significantly more rapidly than normal patients after oral ingestion of 20 mg cholyl[1-^{14}C]glycine (*49*). The study is being repeated with ^{13}C. The path traversed by the labeled substrate before labeled CO_2 is exhaled is generally complex and one can anticipate that the curves showing the appearance of excess labeled CO_2 in the breath of diseased and normal subjects frequently will not be clearly separated. Figure 8 shows the rate of appearance of excess ^{13}C in breath CO_2 of diabetic and normal human subjects who ingested a standard load of glucose uniformly labeled with ^{13}C (*50*). The data suggest that the metabolic defect in these diabetics is the inability to discontinue gluconeogenesis after glucose ingestion, a possibility that can be evaluated with ^{13}C-labeled pyruvate or lactate (*50*).

The assay of metabolites other than CO_2 for stable isotopes by sophisticated mass spectrometry and NMR techniques should have many applications in biomedical research. Figure 9 shows a sequence of ^{13}C NMR spectra obtained from a suspension of live *Candida utilis* yeast cells treated with [1-^{13}C]glucose. The point of interest is that the metabolism of a substrate specifically labeled with ^{13}C can be determined in vivo by direct measurement of the complex mixtures, no tedious and time-consuming separations and degradations being required (*34*). The combined techniques of gas chromatography and mass spectrometry show great promise in studies of the metabolism of substances labeled with stable isotopes. For example, it has been shown that the drug nortryptyline, when administered as a mixture (ratio 1 : 1) of the natural substance and the ^2H- or ^{15}N-labeled material, can be detected in crude urine extracts at the nanogram-picogram level (*51*).

Similarly, ^{13}C-labeled glycine and gas chromatography–mass spectrometry are being used in a detailed study of patients exhibiting hyperglycinemia and the Lesch-Nyhan syndrome, the latter being manifested by an abnormal metabolism of purine (*52, 53*). Patients exhibiting hyperglycinemia convert [1-^{13}C]glycine and [1-^{14}C]glycine to CO_2 much more slowly than normal individuals. Patients with the Lesch-Nyhan syndrome vastly overproduce [^{14}C]purine (and uric acid) from uni-



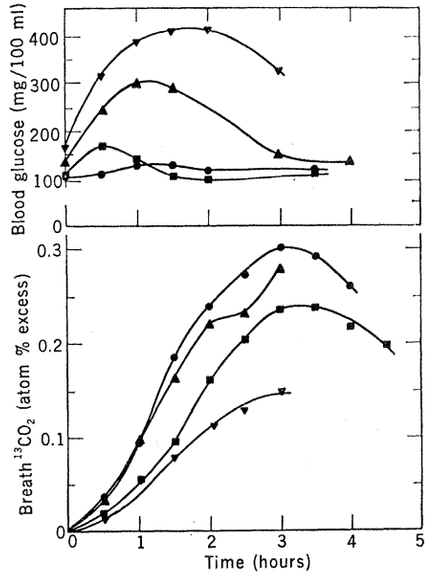


Fig. 7 (left). The rate of appearance of excess ^{13}C CO_2 of the breath of a human being after oral ingestion of an aqueous solution of [1,2-^{13}C]acetic acid (90 percent) containing 700 mg of excess ^{13}C (*48*). (Atom percent excess ^{13}C, atoms of ^{13}C in excess of natural abundance, expressed as a percentage.) Fig. 8 (right). Concentrations of total glucose in the blood (mg/100 ml) and ^{13}C-labeled CO_2 in the breath of subjects after oral administration of 40 g/m^2 (of body area) of glucose uniformly labeled with ^{13}C at 1.0 atom percent excess ^{13}C: ▼, severe diabetic; ▲, mild diabetic; ■ and ●, control (nondiabetic) subjects.

formly labeled [^{14}C]glycine. In Fig. 10, data are compared for the incorporation of ^{13}C-labeled glycine and ^{14}C-labeled glycine into urinary uric acid by a diseased patient (*52*). Two aspects of the data are especially noteworthy. First, there is a close correspondence between the incorporation of a single ^{13}C atom and the incorporation of radioactive ^{14}C. Second, the mass spectrometric measurements of ^{13}C allow one to distinguish uric acid labeling resulting from incorporation of the intact C–C unit of glycine as opposed to labeling by one carbon unit. In addition, these studies emphasize the importance of experiments being conducted in vivo with human beings; the nonradioactive [^{13}C]nuclide is especially suited to such experiments. Experiments in vitro with fibrinoplasts and erythrocytes failed to reveal differences between hyperglycinemics and normal subjects (*53*).

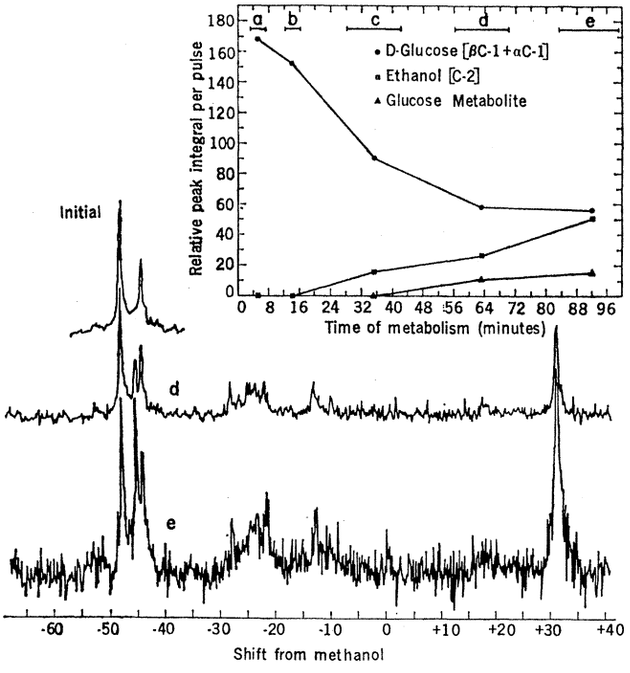
Biological Effects of Stable Isotopes

The occurrence of carbon-13 isotope effects in biological systems has been recognized for some time. For example, the ratios of ^{13}C to ^{12}C in marine limestones are larger by about 25 parts per thousand than those in marine petroleums (*54*). This small isotopic fractionation is the result of cumulative isotope effects on a number of processes, for example,

$$CO_2(\text{gas}) \rightleftarrows CO_2(\text{aq.}) \rightleftarrows CO_2(\text{intracell.}) \rightleftarrows \text{intracellular reduced carbon} \rightarrow \text{oils}$$
$$HCO_3^-(\text{aq.}) \rightleftarrows \text{solid carbonates}$$

Several workers found that algae, like other green plants, discriminate between the isotopes of carbon during photosynthesis (*42, 55*), assimilating $^{12}CO_2$ about 3 percent more rapidly than $^{13}CO_2$. This isotope effect is, at least in part, a manifestation of events at the enzyme level. It has been established that plants which incorporate CO_2 through ribulose-1,5 diphosphate carboxylase and other enzymes of the Calvin cycle are enriched in ^{12}C with respect to atmospheric CO_2 and that the ratios of ^{13}C to ^{12}C in tropical grasses are somewhat higher than those in plants possessing only a Calvin cycle (*56*). In addition, small ^{13}C isotope effects (< 2 percent) have been found in the enzymatic decarboxylation of oxaloacetic acids in vitro (*57*).

On this basis one would not anticipate adverse biological effects being caused by stable isotopes used in small amounts. Even so, the biological effects of the incorporation of large amounts of stable isotopes in biological systems are of importance in medical and environmental studies. When large quantities (> 1 kg) of the alga *Chlorella pyrenoidosa* (*58*) and the yeast *Candida utilis* (*59*) were grown in the presence of CO_2 and acetate, both labeled more than 90 percent with ^{13}C, no adverse effects of the heavy isotope were detectable in growth rates, generation times, or gross cell morphology. With each organism, the cells rapidly attained about 98 percent of isotopic equilibrium with the substrate; the approximate 2 percent disparity might have been due to analytical errors but is well within the range of biological effects observed with ^{13}C. However, in an earlier study, the detailed cytological examination of *Chlorella vulgaris* cells enriched with ^{13}C on a smaller scale (about 90 percent) suggested that ^{13}C in large amounts does affect cells biologically, and in particular may affect the thickness of cell walls and the cellular carbohydrate content (*60*). The results of this study also suggested



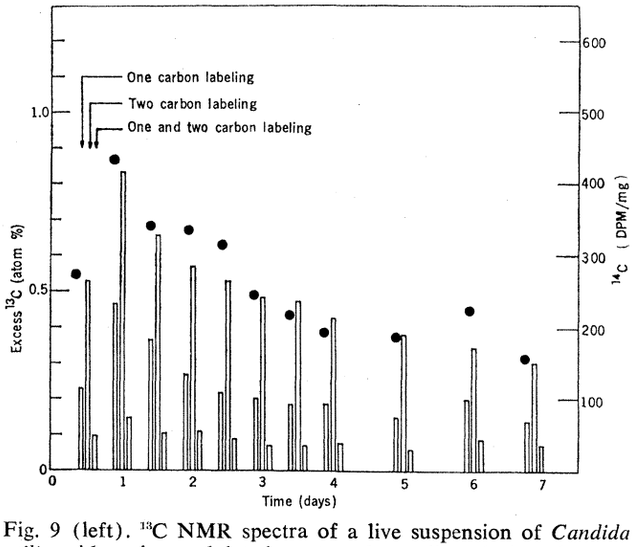


Fig. 9 (left). ^{13}C NMR spectra of a live suspension of *Candida utilis* with noise-modulated proton decoupling during the metabolism of [1-^{13}C]glucose: (a) 3 to 7 minutes after the initiation of metabolism; (b) 12 to 16 minutes; (c) 28 to 42 minutes; (d) 56 to 70 minutes; (e) 83 to 99 minutes. The spectra obtained during the initial time period show only the signals of the C-1 carbon atoms of the α- and β-anomers of extracellular glucose. The signal at about 32 ppm is due to the C-2 carbon atom of product ethanol enriched with ^{13}C whereas that at about 45 ppm is probably due to intracellular glucose or [1-^{13}C]glucose 6-phosphate. Signals in the region between -12 and -28 ppm arise from natural abundance ^{13}C in glucose carbon other than C-1. [Courtesy *FEBS Lett.* (*34*)] Fig. 10 (right). Excretion isotopes in urinary uric acid following administration of [1,2-^{13}C]glycine (50 mg/kg) (90 atom percent excess) and uniformly labeled [1,2-^{14}C]glycine (2 μc) (●) to a patient with Lesch-Nyhan syndrome (*52*). Urine was collected every 12 hours and uric acid was isolated by crystallization, and was purified by chromatography on Sephadex G-10. Isotope ratio measurements were made at M+1/M (225/224), M+2/M (226/224) and M+3/M (227/224) with the use of the gas chromatograph, mass spectrometer, alternating voltage accelerator system previously described (*62*); M represents the mass of the parent ion and (M+1), (M+2), and (M+3) represent ions in which one, two, or three ^{12}C are replaced by one, two, or three ^{13}C. [Courtesy Nyhan and Klein (*63*)]

that a complex pattern of cellular effects may accompany multiple substitution with ^{13}C, ^{15}N, ^{18}O, and especially ^{2}H.

Studies of the effects of incorporation of large amounts of ^{13}C in more complex biological systems are now in progress. A potato plant grown in a closed chamber with CO_2 (in which 31 percent of the carbon atoms were ^{13}C) as the sole source of carbon has produced 1.7 kg of tubers with carbohydrates in which 29 percent of the carbon atoms are ^{13}C (*14*). It is significant that the distribution of the ^{13}C label among the six carbon atoms of starch is uniform and that the plant and tubers were indistinguishable on gross examination from those grown with normal isotopic CO_2. Two weanling mice were kept on a diet of *C. utilis* yeast containing about 90 percent ^{13}C, supplemented with small amounts of natural vitamins, minerals, sulfur amino acids, and roughage for approximately 6 months (*10*). During this time their weight gain was normal and their body carbon content increased to about 60 percent ^{13}C. Unfortunately, one of the mice died after 4 months because of accidental asphyxiation. The study on the other mouse was terminated voluntarily at 6 months; no adverse effects of the ^{13}C incorporation were apparent from gross pathological examination of tissue samples of either animal. The ratios of ^{13}C to ^{12}C in body fluids and tissue samples are being determined and the results should be of some significance because the ^{13}C enrichment data, when compared with the known turnover rates for carbon in various tissues, may permit an evaluation of the metabolic rates in situ of the major organs and tissues. We hope that more detailed and quantitative work in this interesting area of biological effects of heavy isotopes will be conducted, particularly with regard to the effects of such isotopes upon the physiology and reproductive potential of higher plants and animals.

Conclusions

Within the last few years, the quantities of the separated stable isotopes of carbon, oxygen, and nitrogen in a variety of chemical forms have been increased dramatically. The analytical techniques for the detection and assay of stable isotopes have also become increasingly refined and more widely available. Both these developments have led to a sharp increase in research with stable isotopes in the life sciences and medicine. As production of stable isotopes increases and instrument development continues, this trend can be expected to accelerate.

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64. We are indebted to the late Dr. Eugene S. Robinson who was the prime force in the development of the stable isotopes program at Los Alamos. We thank Professors B. N. Tolbert and L. O. Morgan for helpful criticism and suggestions and Drs. C. T. Gregg, B. B. McInteer, W. W. Shreeve, G. A. Cowan, and G. Voelz for valuable discussions. The following publications on stable isotopes in science and medicine are recommended: P. D. Klein and J. L. Roth, Eds., *Proceedings of a Seminar on the Use of Stable Isotopes in Clinical Pharmacology, University of Chicago, 1971* (available from National Technical Information Service, Springfield, Va.), U.S. Atomic Energy Commission Conf. 71115; D. G. Ott, Ed., *Proceedings of the First National Symposium on Carbon-13*, Los Alamos, N.M., 1971 (in press); G. Jung and F. Jütner, *Chemiker-Zeitung* **96**, 603 (1972). These last authors discuss briefly some of the points we emphasize herein but our article is largely complementary to theirs; of particular interest are their discussions of mass culturing of algae and the applications of ^{15}N. Our work was performed under the auspices of the U.S. Atomic Energy Commission.

Specific Suppression of Immune Responses

Antibody directed against either antigen or receptors for antigen can suppress immunity specifically.

Donald A. Rowley, Frank W. Fitch, Frank P. Stuart, Heinz Köhler, Humberto Cosenza

A fully responsive immunologic system is essential for survival against encounter with infectious, oncogenic, and toxic agents; however, in particular instances, an immune response may be disadvantageous. Allergy to a common pollen, maternal antibody that causes erythroblastosis in the fetus, and immunologic rejection of a life supporting organ transplant are familiar examples of undesirable responses. Immune reactions, abnormal in magnitude or kind to certain infectious or other agents, apparently are involved in various "autoimmune" diseases affecting diverse tissues or organs (*1*). Antigen-antibody complexes may localize in the kidney causing renal injury (*2*). Furthermore, antibody to tumor antigens may prevent cell mediated immunologic injury to the tumor; thus, suppression of the antibody response to a tumor may be desirable (*3*).

The general approach for preventing or controlling life threatening immune reactions has been to reduce the total number of lymphoid cells, usually by administering cytotoxic drugs or irradiation or, by giving heterologous antibody directed against lymphoid cells (*4*). These procedures, which nonspecifically suppress responses to all antigens, are sometimes remarkably effective in promoting graft survival or controlling autoimmune processes. However, the complications of immunosuppressive therapy are frequent and often severe; they include overwhelming infection, increased risk of malignancy, and injury to nonlymphoid tissue (*5*).

Ideally, immunosuppressive therapy should selectively suppress either the antibody or the cell mediated response to a specific antigen without altering responses to other agents. This ideal has been achieved clinically in one instance. Immunization by fetal Rh antigen is prevented by giving the Rh negative mother antibody against Rh antigen immediately after she has given birth to an Rh positive child (*6*). The mechanism of specific suppression achieved by giving antibody passively has been studied extensively (*7*). Other studies demonstrate that specific regulation can be achieved by

Drs. Rowley and Fitch are professors in the department of pathology, Dr. Stuart is associate professor in the department of surgery, Dr. Köhler is associate professor in the departments of pathology and biochemistry, and Dr. Cosenza is an assistant professor in the department of pathology at the Pritzker School of Medicine and the Division of Biological Sciences, University of Chicago, Chicago, Illinois. The present address of Drs. Rowley, Köhler, and Cosenza is La Rabida Research Institute, University of Chicago.