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**PROCEEDINGS OF SEMINAR ON THE USE OF STABLE
ISOTOPES IN CLINICAL PHARMACOLOGY,
UNIV. OF CHICAGO, NOVEMBER 10-11, 1971**

August 1972

The University of Chicago
Chicago, Illinois

Argonne National Laboratory
Argonne, Illinois

and

Los Alamos Scientific Laboratory
Los Alamos, New Mexico

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PROCEEDINGS OF A SEMINAR
ON THE USE OF STABLE ISOTOPES
IN CLINICAL PHARMACOLOGY

November 10-11, 1971

The University of Chicago
Center for Continuing Education

Edited by Peter D. Klein and Lloyd J. Roth

Editorial Coordination
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Co-sponsored by
The University of Chicago
Argonne National Laboratory
and

Los Alamos Scientific Laboratory
and supported in part by the National Science Foundation
and the NICMS of the National Institutes of Health

CONFIDENTIAL

14

Organizing Committee

Lloyd J. Roth, Chairman

Peter D. Klein

Eugene S. Robinson

Bert N. Tolbert

These Proceedings are dedicated
to

Eugene Sant Robinson

May 31, 1916 - June 22, 1972

Biemann

Berlin

Bush

DeGrazia

Fales

Freese

Gaffney

Goldstein

Hofmann

M. C. Horning

Kipnis
iv

Klein

Lester

Matwiyoff

Morgan

Nyhan

Ott

Robinson

Roth

Tolbert

Wall

Watson

Weiger

Photographs by E. S. Robinson
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INTRODUCTION

Lloyd J. Roth
Department of Pharmacology
University of Chicago, Chicago, Illinois

Welcome to the Seminar on the Use of Stable Isotopes in Clinical Pharmacology.

This meeting, organized by Peter Klein, Eugene Robinson, Bert Tolbert, and myself, grew out of a National Symposium on Carbon 13 held at Los Alamos on June 9-11, 1971 which was concerned largely with physical and analytical measurements. At that time it became apparent that a second meeting concerned with biological applications was desirable because the availability of stable isotopes would soon make possible many studies in the human which heretofore could be carried out only with radioactive tracers.

There is no doubt that the introduction of stable isotopes in clinical research will open a new era not unlike that which followed the distribution of radioactive isotopes on a commercial scale two decades ago.

The group here assembled is a heterogeneous one. Everyone is an expert in some area, but lacking expertise in another. Thus, in the best of all possible worlds, each will be able to participate in both the teaching and the learning process. It is in this spirit that we expect this gathering to provide the basis for a continuing dialogue between the participants long after this short contact.

Dr. Robinson's paper will open the meeting. It is a great pleasure for me to introduce him, because Robbie and I were graduate students in chemistry at Columbia University so long ago it is hard to remember. We went in divergent paths, and to come together at this meeting is a personal, if not a slightly emotional, experience for me.

CHEMICAL RESOURCES

Chairman: L. J. Roth

PRODUCTION OF STABLE ISOTOPES

E. S. Robinson

ORGANIC SYNTHESIS AND BIOSYNTHESIS

D. G. Ott

PRODUCTION OF STABLE ISOTOPES

Eugene S. Robinson
Chemistry and Nuclear Chemistry-4
Los Alamos Scientific Laboratory, Los Alamos, New Mexico

One thing that we at Los Alamos have discovered about isotopes is that if they are essentially free, or cheap, people will do experiments with them. If isotopes are kept at high prices, you may buy a gram at \$1,000.00, but it sits on the shelf waiting for the definitive experiment, and no real use is made of it.

Stable isotopes are now being produced at the Los Alamos Scientific Laboratory due to the efforts of Dr. B. B. McInteer and his associates. The techniques they use involve cryogenic distillation of liquid carbon monoxide and liquid nitric oxide.

A typical system as developed by Dr. McInteer is shown in Figure 1. The carbon monoxide plant is in three sections, a single column of 80 feet for the enriching section at the bottom, a combination of 4 columns about 20 feet long, and an upper section of 12 columns 40 feet long, so that there is a total of some 140 feet in the entire plant. It has a production rate of about 4 kg per year, and the feed rates are given in Figure 1. Input is 3020 liters a day which produces about 20 liters a day of enriched material.

In new production facilities now under construction, the unit will be 720 feet high and will include nitric oxide columns.

The NO plants are somewhat more complicated (Figure 2) because ^{17}O must be separated from both ^{16}O and ^{18}O ; also ^{14}N and ^{15}N are both present. A number of different combinations of columns is required. I am just giving you a rough idea of what the production facilities are like. A handout which gives much more detail about what is going on at Los Alamos is available (Los Alamos Scientific Laboratory Report, LA-4391).

What we are doing now and plan to do is probably more interesting to you.

TABLE I
ICONS PRODUCTION

	<u>kg/yr</u>	
	<u>Now</u>	<u>1974</u>
^{12}C	9	156
^{13}C	9	50
^{14}N	180	2600
^{15}N	0.7	10
^{16}O	200	3000
^{17}O	0.08	1.1
^{18}O	0.47	6.8

With such large amounts of stuff available you may wonder what we are going to do with all of it. I think I can give you a rough idea.

Some of the uses of these isotopes are shown in Table II.

TABLE II

USES

1. SNAP REACTORS
2. HEART PUMPS
3. HEART PACERS
4. ^{17}O CHEMICAL RESEARCH via. N.M.R.
5. ^{15}N HEAVY ION ACCELERATORS
6. ^{13}C CHEMICAL RESEARCH - N.M.R.
7. ^{13}C WILL REVOLUTIONIZE BIOLOGY, BIOCHEMISTRY, AND CLINICAL MEDICINE.

^{16}O is being used in SNAP reactors. Those are power reactors in which the heat produced by alpha particles is used. If human beings are working near them one must worry about the neutron background from (α, n) reactions, so they make ^{238}Pu oxide with ^{16}O which has as low a background as the metal itself. It is also used in heart pump experiments as a power source, as well as in heart pacers.

Essentially all of the ^{17}O is used at Los Alamos for NMR in physical, inorganic, and organic chemistry research. No ^{17}O from this source is available outside the laboratory. ^{15}N and ^{18}O are used in chemical research, but they are also used in heavy ion accelerators because they are rich in neutrons. ^{12}C , not listed in the table, will be used for beam dumps and making mechanical parts for accelerators. It has very low cross-sections for many accelerator reactions.

^{13}C is used in chemical research. We also think that because carbon forms the backbone of all organic molecules, it will cause a little revolution in biochemical and medical research.

TABLE III
SOME DISEASES THAT MAY BE DIAGNOSED
BY A SIMPLE ORAL FEEDING OF A SPECIAL COMPOUND OF ^{13}C
AND A BREATH ANALYSIS FOR $^{13}\text{CO}_2$

EARLY DIABETES
THYROID DISEASE
THIAMINE DEFICIENCY
STEATORRHEA
FOLIC ACID DEFICIENCY ANEMIAS
RADIATION SICKNESS
HYPER-ADRENAL GLUCOCORTICOIDISM
PELLAGRA
DIVERTICULITIS
TROPICAL SPRUE
MALABSORPTION SYNDROME
HYPERLIPEMIAS
GOUT OR HYPERURICEMIA
ARTEROSCLEROSIS
HEART DISEASE

Table III was compiled by Dr. Walton Shreeve from Brookhaven National Laboratory. He suggests a group of diseases that may be diagnosed by simple oral feeding and breath analysis for ^{13}C dioxide. In fact, there is a cooperative experiment on the diagnosis of early diabetes among Los Alamos Scientific Laboratory, Brookhaven National Laboratory, and the Argonne National Laboratory, in which Dr. Peter Klein is taking part.

With these isotopes available in large quantities, it is desirable to have simple and convenient schemes for detection. Such methodology is all-important for you who need to use them for diagnostic purposes or for tracing. Methods that are available to you, or at least some of them, are presented in Table IV.

TABLE IV

METHODS OF DETECTION

MASS SPECTROSCOPY - MOST SENSITIVE AND GENERAL METHOD. DIFFICULT WITH COMPLICATED MOLECULES.

NUCLEAR MAGNETIC RESONANCE - ODD NUCLEI ^{13}C , ^{15}N , and ^{33}S CAN "SEE" THE ENVIRONMENT OF THE ATOM IN A COMPLEX MOLECULE.

VIBRATIONAL SPECTROSCOPY - SIMPLE MOLECULES, INFRARED AND RAMAN SPECTROSCOPY.

ROTATIONAL SPECTRA - SIMPLE MOLECULES, INTERFEROMETER + FOURIER TRANSFORM

Mass spectrometry is one of the most sensitive and general methods. However, for very complex molecules it is a lot of work to get the information out in usable form.

Nuclear magnetic resonance, a newer technique, offers the advantage that you can actually see the site of the ^{13}C so that structures may be obtained, which is more difficult by other techniques. In mass spectrometry the site of ^{13}C incorporation must be found by degradation of the molecule. The odd nuclei, such as ^{17}O , ^{13}C , ^{15}N , and ^{33}S , are active in nuclear magnetic resonance.

The disadvantage of nuclear magnetic resonance is that one needs at least 10^{20} magnetic nuclei in a sample of about 2 ml, so that it requires more material than does mass spectrometry. In experiments with human beings, they being large animals, a large sample may be taken; microscopic amounts are not involved unless you are interested in substances of very low abundance.

Vibrational spectroscopy can also be used, but the sensitivity is somewhat lower. Isotope ratios may be determined to about 1.5%. In some cases, the infrared spectrometer may be capable of doing breath analyses for ^{13}C in CO_2 .

H. A. Gebbe, from the Bureau of Standards at Boulder, Colorado, has developed very sensitive rotational spectrometric techniques. Samples must be converted to something that has a good rotational spectrum. Carbon dioxide will probably have to be reduced with zinc dust to carbon monoxide in order to get a decent measurement.

A type of measurement not listed in Table IV uses reactions that can be carried out at institutions where accelerators are available. Reactions like the ^{13}C (p, γ) and ^{12}C (p, γ) may be exploited. These are extremely sensitive. Isotope ratios may be obtained with as little as 10^{-9} grams of material.

One of the things about ^{13}C , and other separated isotopes, used for tracing is their apparent lack of toxicity. In the case of the hydrogen isotopes, with a mass ratio of 2.00, a real chemical isotope effect is observed, and, indeed, deuterium compounds are toxic at high concentrations. However, for carbon isotopes the ratio of masses is only 1.08, so you don't really expect to see a significant rate change in chemical reactions that would affect the growth of an animal. Our experience at Los Alamos is that ^{13}C does not seem to be toxic, at least to small mammals. I will leave the problem of toxicity to be discussed later in the meeting.

Figure 1. Production of ^{13}CO by cryogenic distillation.

Figure 2. Production of ^{15}NO by cryogenic distillation.

DISCUSSION

Dr. Berlin: There is one question I think many of us are going to face in one way or another; perhaps at this stage it would be good if you would give us some order of sensitivity for detection compared to radioactive isotopes. You may also want to tell us a little bit about the mass of material that you are going to need to achieve this.

Dr. Robinson: Clearly, techniques for the radioactive isotope are much more sensitive than those that can be used with the stable isotopes; they have essentially zero background in all cases. While multilabeling will enhance the sensitivity for stable isotope detection, you can't come up with a sensitivity of that of carbon-14 in an experiment. The amounts of material you use, I think, are really going to be dependent on the type of experiment. One of the things that you will realize is that you can use large amounts of these isotopes in an experiment, whereas you cannot use large amounts with the radioactive material.

Dr. Berlin: My question is the mass of material that you have to start with for analysis. Do you need a gram or 0.1 gram?

Dr. Robinson: Clearly, I think with mass spectrometry it would be in the order of micrograms of material, and still you would get good analyses. In fact, I think people are doing that routinely around the country. Klaus Biemann might be a good man to ask what the sensitivity of mass spectrometry is for isotopes at this point.

Dr. Biemann: That will be discussed at great length this afternoon, it very much depends on whether you talk about a pure sample or if you talk about a blood serum sample in which you have 0.1% of labeled material.

Dr. Goldstein: In proton magnetic resonance spectra, sensitivities down to 10 μg have been obtained at 90 megahertz, and with the trend toward high frequencies, that will improve. It may be that single microgram quantities can be used, not only for detection but also for characterization. As Dr. Robinson pointed out, this will be very sensitive to purity and will also be very sensitive to the general conditions. One microgram may be sufficient.

Dr. Robinson: One of the very nice things about nuclear magnetic resonance is that you don't have to clean up the sample very much. You can use a rather crude solution, whereas with mass spectrometry you have to clean things up a bit. Actually, you can use NMR for blood samples, serum, urine, and that sort of thing directly, which is hard to do with mass spectrometry. You have to do some cleaning up.

Dr. Hoffman: You have given us a five-fold increase in the production of carbon-13. Is there any reason to believe that this increase cannot continue and that you will keep making more and more? There is nothing that prevents that except manpower and money and equipment, is there?

Dr. Robinson: I think that is true. We will be using two holes in our new building. We have space for twelve holes, so in this facility you could expand production by a factor of five or six.

Dr. Borg: It would also seem fair to say that if, from the beginning of practical application, there is a wide interest and a wide market, and there is profit to be made, commercial producers will start production facilities.

Dr. Jenden: Can you tell us anything about the possibilities of neutron activation analysis for specific detection of stable isotopes?

Dr. Robinson: Yes. With oxygen isotopes you can use the following ones: $^{16}\text{O} (n,p) \rightarrow ^{16}\text{N} + \beta$, and with $^{17}\text{O} (n,p) \rightarrow ^{17}\text{N} + \beta$. With $^{18}\text{O} (n,\alpha)$ you get carbon-15 with β decay. Those reactions, though, are not quite as sensitive as this one involving, say, the $p-\gamma$ or $p-\alpha,\gamma$ reactions that I mentioned earlier, although you do get a sample that you can count.

Dr. Tolbert: Perhaps I could enlarge on the question that Dr. Jenden has asked. There was a study made at the Oak Ridge National Laboratories on the detection of carbon-13 ratios by an accelerator bombardment technique, and they used 3 MeV particles. There is a large number of reactions that can be used, but in almost all cases an energy range of 1 to 20 MeV is necessary. That means, in general, in the particle activation processes, a large, expensive machine is needed. The long-term prospect for activation processes is not optimistic; in fact, they are rather pessimistic, and I believe the Oak Ridge people have abandoned their carbon-12 and carbon-13 work. They could determine the overall carbon-12/carbon-13 ratio but with no better accuracy than burning the sample to CO_2 and putting it in the mass spectrometer. That is a lot cheaper than putting it in the accelerator.

Dr. Robinson: Some of the experiments that have been done on these ratios at Los Alamos were not done with our big accelerators but were done with a rather small--I won't say portable, but small--Van de Graff accelerator. What the cost is, I don't know; but perhaps in many cases you don't need the large, expensive machines to actually carry out experiments. We compiled a collection of papers and reports of experimental work at Los Alamos on this subject; if you are interested in this compilation you can let me know and we will see that you get a copy of it.

Dr. Nyhan: Could you expand on the question of toxicity that you raised? I don't know anything about deuterium toxicity. What happens to the animal?

Dr. Robinson: People who are to speak this afternoon will talk about that in detail, but we had two mice being fed a diet of 95% carbon-13 and they survived for a considerable time. One died, apparently of natural causes. There didn't seem to be anything in the autopsy which indicated that carbon-13 was involved.

Dr. Nyhan: I think I have the picture on the carbon-13, and it is reassuring. What about the deuterium?

Dr. Robinson: As far as I know, we haven't raised any mice on deuterium at Los Alamos, but deuterium is definitely toxic; in fact, I think, with the rat, when the environment is about 30% deuterium, it gets to be a pretty slow animal.

Dr. Sweeley: This afternoon I will show some experimental data on a human metabolic experiment where we infused 30 grams of pure dideuterio glucose into a normal volunteer and a patient. This had absolutely no effects we could see, in following the two individuals for some time. At the level where you might really want to do a metabolic experiment I don't think deuterium is toxic.

Dr. Klein: Dr. John Thomson in our division has done considerable work on deuterium toxicity. What happens is that by the time you reach 30% D₂O in the body water the animal will die, and probably it is because all the enzymes come to a grinding halt because they can't pluck off those hydrogens. The enzymes get slower and slower, and that is probably one of the major causes of death.

Dr. Wall: There is a practical point involved which may over-emphasize the toxicity of deuterium. In actually labeling compounds, most of the drugs are going to have a molecular weight of at least 150 to 200. Usually only two deuteriums will be put in that molecule, so there is all the difference in the world between the actual case of a labeled compound and that where 30% of body water is D₂O.

My point is that emphasizing the toxicity of these high levels that have been alluded to is unrealistic. One is unlikely in practice ever to have deuterium at these levels.

ORGANIC SYNTHESIS AND BIOSYNTHESIS

Donald G. Ott
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It is obvious that preparation of labeled compounds is totally dependent on the availability of the isotopes in enriched concentrations; it should also be apparent that utilization of the enriched materials from a production facility is almost totally dependent upon conversion of its output to other chemical forms. This is true whether the nuclides involved are stable or radioactive (I am, of course, referring primarily to components of organic compounds, and ICONS in particular (Isotopes of Carbon, Oxygen, Nitrogen, and Sulfur). Some studies can be made in which the oxides from the enrichment process enter the systems under study directly (such as, tracers in the atmosphere and reactions of metal carbonyls) and some uses involve conversion to inorganic compounds for physical chemistry and molecular structure studies. Clearly, however, the bulk of the requirements involve incorporation of the enriched isotopes into organic compounds. The needs are for all degrees and combinations of labeling -- some compounds must be uniformly (equally) labeled, some specifically labeled in one or more particular positions, and some multiply labeled with more than one isotope, both uniformly and specifically. For some uses high enrichment is required; for others low enrichment is necessary (or sufficient). The quantities of compounds needed can vary from milligrams to kilograms. The methods of choice include biosynthesis for uniformly labeled complex materials, organic chemical synthesis for specifically labeled compounds, or a combination of biosynthesis followed by organic chemical conversions and modifications of the products.

This morning I'd like to consider some of the unique aspects and problems encountered with stable isotopes, tell you of some of our syntheses with ^{13}C , and indirectly indicate some of the applications we are seeking and contemplating for the ICONS at Los Alamos. And when I say "we" I mean various members of our staff, and, particularly with respect to biosynthesis, Dr. C. T. Gregg -- one of the participants at this seminar.

Syntheses of isotopically labeled compounds merely make use of reactions and techniques of organic and biochemistry. But -- there are several considerations which make it a different game than conventional synthesis; or, if it is the same game, it's played by a different set of rules. The individual parts of a labeling scheme

are almost always quite conventional in themselves; it is the way they are put together that often makes isotopic syntheses differ from the normal approaches and requires the chemist to think perhaps a bit strangely about alternative routes to a desired product.

There are various considerations which necessitate this. One, of course, is isotope economics. The cost and availability of the isotope are usually a prime consideration in developing a procedure to give a high yield of product based on the isotopic starting material. In addition to this being a very practical sort of thing, it is also usually aesthetically pleasing to the chemist. However, in seeking this pleasure, the overall economics may go to pot. For example, getting the yield up from 85 to 95% by spending an extra week playing with reaction conditions can be relatively costly if it is a one-shot prep that is not too big. That is, saving a hundred dollars worth of ^{13}C on a 1-mole prep by spending hundreds more in salary, overhead, and materials is not always the way to go. A couple of years ago though, considering the price of ^{13}C then, it would have been well worth it. Also, if the reaction is to be used on a large scale or repeatedly, working as hard as possible for conservation of isotope is certainly the thing to do, but the chemist should not neglect to evaluate whether the isotope may not be the cheapest thing in his pot. Sometimes, of course, it is not simply the purchase price of the isotope or the isotopic starting material -- it is its availability -- that makes a high yield desirable.

Another consideration that makes syntheses with isotopes a little different is that starting materials and reactions that are best for the specific purpose can be those that would never be considered practical for preparation of the unlabeled material itself. This is independent of economics per se, it being the only way to arrive at the desired product. Economic considerations are always present, of course -- be they those of price, time, personnel, or materials availability.

The varied nature of the requests for labeled compounds and the consequent variety of reactions are some of the factors that make things a little different, as well as very interesting, for those of us involved in syntheses with isotopes. Quite a few of us here in this auditorium don't find it too difficult to recall the days when ^{14}C and tritium labeling were getting going as the starting materials became readily available. I think the analogy is readily drawn, that with stable isotopes it is the same story all over again. The catalogs with the hundreds of ^{14}C and tritium-labeled compounds weren't with us 15 or 20 years ago; 15 or 20 years from now there will, no doubt, be catalogs with almost any compound you want labeled with ^{13}C , ^{15}N , ^{17}O or ^{18}O , ^{33}S , or whatever. Actually, there is a start now on the more basic materials, and these suppliers are increasing the number of items as fast as they can.

Although there is a real similarity between the early days of ^{14}C labeling and the period we are now entering of synthesis of compounds labeled with ^{13}C , there are different synthetic problems. These

differences are primarily because of the difference in scale of the preparations. Usually the ^{13}C preps are considerably larger; sometimes this makes things easier -- sometimes it doesn't. Many of the elegant schemes developed for ^{14}C are not applicable to ^{13}C at all -- they simply don't scale up.

A good example of this was our first organic synthesis requirement -- large-scale preparation of doubly-labeled acetic acid. (I should mention that "labeled," unless I indicate otherwise, means ^{13}C at around 90 atom %.) The acetate was to serve as the sole carbon source for growth of the yeast, *Candida utilis*, which in turn, was to be the complete diet for mice. Although no one who has given the problem much thought believes that ^{13}C is toxic even at high levels, it was felt that clinical applications would be encouraged by demonstrating this in the mouse. I'll not discuss the mouse feeding experiment further at this time, except to say that no effects attributable to ^{13}C were noted after several months of feeding, and this phase of feeding experiments has now been terminated. Anyway, this was how the requirement for kilogram amounts of ^{13}C acetic acid arose. Well -- those of you who have practiced the black art of organic chemistry realize that the methods which have served so admirably for ^{14}C acetic acid, such as the Grignard reaction and others, would be very impractical on this scale.

The method chosen and which we are quite pleased with was synthesis of methanol by hydrogenation of carbon dioxide followed by carbonylation of the methanol with carbon monoxide.

Paulik and Roth,
Chem. Commun., 1578 (1968)

The preferred route to methanol is hydrogenation of carbon dioxide -- carbon monoxide works fine, too, although the activity of the catalyst does not remain so constant.

Davies and Snowden,
U. S. Patent 3,326,956 (1967)

Figure 1. is a schematic of the apparatus used routinely to produce 5 or 6 moles of methanol in a days time. The system volume is around 20 l -- the catalyst bed is about 400 ml.

Acetic acid is, of course, a generally useful synthetic intermediate. One such use is illustrated in Figure 2. You'll be hearing more about such a labeled steroid later.

For preparation of bromoacetic and ethyl bromoacetate, we prefer to first convert at least part of the acid to the anhydride.

The three isotope isomers of acetic acid have been used to produce the corresponding EDTA's (equation 8) and the old classical Gabriel reaction for the three glycines. This reaction, of course, is applicable to the ^{15}N - ^{13}C combinations as well.

The ready availability of methanol afforded formaldehyde (another generally useful synthetic intermediate) which has been used to prepare cysteine-3- ^{13}C (equation 11) which will be used to biosynthesize ferredoxin for NMR studies of its structure at the active site.

Ring-labeled histidine-2- ^{13}C has been prepared for NMR investigations of the structure of hemoglobin in solution (equation 12).

(12)

Cyanide for this prep was prepared by a somewhat round-about, but sure-fire, method and we are currently developing a more satisfactory way of making HCN from carbon monoxide. Preparation of HCN, although quite a simple molecule and a very valuable synthetic intermediate, is another example of the inability of an elegant ^{14}C method to scale up.

(13)

The L-histidine was prepared from cyanide using the method developed for ^{14}C in which the starting material is L-histidine.

A more convenient route for cyanide, particularly on a large scale and with consideration of the useful intermediates generated,

is shown in equation 14. The last step has given us trouble and it is likely that we'll actually be using p-toluenesulfonyl chloride for dehydration of the formamide.

Glucose-1-¹³C may also involve cyanide through the old standard Kiliani-Fischer synthesis; however, we are taking a little time to look at the possibility of a novel method involving a Wittig reaction.

The glucose is needed for initial clinical trials of ¹³C as a diagnostic tool to be undertaken around the first of the year with Walton Schreeve from Brookhaven National Laboratory who will be spending the first half of next year at Los Alamos.

As the capability is achieved for the synthesis of a labeled compound, the capability is increased for the production of others. As an example, the synthesis of cysteine-3-¹³C necessitated development of formaldehyde synthesis, which, itself, was considerably simplified since we had already developed a methanol synthesis. And now, since we already have a method for formaldehyde, we can consider an alternative, possibly simpler route to glucose-1-¹³C than the well known method involving cyanide.

Photosynthesis is perhaps the simplest of the biosynthetic methods. (Figure 3). Algae (*Chlorella pyrenoidosa*) have been grown in a 5-liter shaker box to give around 5 g per day at 90+ atom % ^{13}C with no observed deleterious effect of the ^{13}C . The chlorophylls have been isolated for NMR studies, the amino acids for use in histone investigations, and the fatty acids for mass spectrometric characterization. Algae will provide the best source of a number of uniformly labeled materials. For larger-scale production, however, *Chlorella* may not be the organism of choice. As some of you know, it's the amount of light that can be forced in which is limiting in algal growth, and it is likely that, in a large fermentor, a blue-green, *Nacystus nidulans*, will have a better growth rate.

The large shaker boxes in Figure 4 are also being used in recently started investigations with a marine alga, a red kelp, for production of uniformly labeled galactose. This, also, will be used in our initial trials of ^{13}C for clinical diagnosis.

Biosynthesis using thalli from kelp or leaves from such things as spinach or tobacco is a way of labeled compound preparation which is somewhat different from that in which materials are isolated from a completely labeled organism like *Chlorella*. Use of leaves or thalli capitalizes on particular characteristics of the system which results in efficient conversion of carbon dioxide to one, or a few, specific compounds. Tobacco leaves are routinely used in the commercial synthesis of uniformly ^{14}C labeled glucose, and the kelp thalli for ^{14}C galactose. The specific activity or atom % excess in the product not only depends on the isotope concentration of the CO_2 , but also on dilution of the product by endogeneous material. With ^{14}C this merely has the effect of lowering the specific activity; but with ^{13}C , if one is trying to produce material at a high concentration of ^{13}C (say 90 atom %), the presence of endogeneous material containing normal-abundance carbon, becomes a very important factor. Also, as I'll mention later, a compound containing (for example) 45 atom % ^{13}C obtained by biosynthesis from 90 atom % CO_2 accompanied by dilution with an equal amount of endogeneous material, is definitely different from the compound obtained from 45 atom % CO_2 and no endogeneous product. Thus, in the development of methods, studies of preincubations, when to dilute (if ever), and so forth, can be quite important.

Small fermentors, such as the 12-liter version shown in Figure 5 are also very valuable for carrying out biosynthesis preps. Owing to the mouse-feeding experiment, we have now had considerable experience in the production of ^{13}C yeast with acetate serving as the sole carbon source. Many labeled compounds can, of course, be isolated from this material. Yeast has the advantage of quite rapid growth -- hundreds of grams per day can be produced in a 200-liter fermentor (Figure 6) using acetate as the carbon source. The disadvantage is the additional effort required for acetate synthesis and also that 60 to 70% of the acetate carbon is expired as CO_2 and must be recovered and recycled through the methanol-acetic acid process (but, of course, recycling of garbage is fashionable these days).

Carrying out syntheses with stable isotopes involves some interesting things that usually aren't encountered -- even with radioisotopes. For example, molecular weights: acetic acid at 90 atom % ^{13}C has a molecular weight of 61.8, not the usual 60. And its specific gravity is 1.08, not 1.05. Methanol is 32 g per mole, not 31. And "proof of identity" by infrared requires additional considerations.

Multiple labeling, in addition to being automatic in photosynthesis or in growth of yeast on acetate, is (among other things) significant for detection efficiency. (One should consider the differences between multiple labeling and uniform, or general, labeling.) Since ^{13}C occurs at about 1% in nature, the probability of two ^{13}C 's occurring adjacent to each other in the same molecule is 10^{-4} , and for three is 10^{-6} , and so on. Multiple labeling decreases the background tremendously and thus increases detection sensitivity -- that is, it does unless the analytical method takes the material back to a one-carbon compound, such as CO_2 . But it must be noted that intermediate levels of overall enrichment can be obtained in different ways. For example -- a rather extreme example -- a doubly-labeled molecule at 20 atom % ^{13}C , with the same enrichment at each of the two positions, can result from two different types of distributions. The material could be synthesized from 20 atom % single-carbon precursors, or synthesized from 100 atom % precursors and then diluted with four parts of ^{12}C compound. The resulting materials would both be 20 atom % ^{13}C , but the distribution of isotopic isomers would be entirely different. In the first case, only 4% of the molecules would be doubly labeled, and 32% singly labeled and 64% would be all ^{12}C . In the latter case, 20% of the molecules would be doubly labeled and 80% unlabeled, with none singly labeled. This makes a tremendous difference to NMR applications because of spin-spin coupling, as well as to the mass spectroscopist. However, analysis for ^{13}C by combustion to CO_2 or in application of ultracentrifugation would give identical results.

Multiple labeling offers a unique advantage of stable isotopes in "finger-printing" of complex molecules. This characteristic is expected to prove quite useful in applications of stable isotopes in industry and as environmental tracers.

The spectra in Figure 7 show the various splittings of the proton resonance by the 1-carbon, and the relative areas of the various peaks can be used to calculate the isotope-isomer composition. The new simple, relatively inexpensive Perkin-Elmer Hitachi ^{13}C -NMR spectrometer was used for these spectra. The point is, considerable information can often be obtained with proton instruments, and the fact that one doesn't have a ^{13}C -NMR machine shouldn't mean he shouldn't be using ^{13}C .

In conclusion I'd like to note that my remarks have necessarily been incomplete and not detailed and have been confined almost exclusively to ^{13}C -- but essentially all the considerations apply to the other stable isotopes as well.

Our primary efforts at present are directed toward demonstrating the utility of stable isotopes for biomedical research and clinical applications. This has necessitated the establishment of a modest synthetic capability which is serving the present requirement of our program and forms the basis for future developments.

A major point which emerges from even a simple consideration of the synthetic requirements which we can undoubtedly expect to arise is that a centralized synthesis facility with a wide variety of tools and techniques at hand for a reasonably efficient solution for each synthetic problem will be necessary. Such a facility will provide a beneficial impetus to future research as well as to commercial development.

And, finally, all of us involved in the Los Alamos ICONS program would be most pleased to visit with you, discuss our problems and methods in detail, and just generally talk about any aspect of our favorite subject.

Figure 1. Apparatus to produce methanol from carbon dioxide and hydrogen.

Figure 2. Incorporation of labeled acetic acid into Ring A of cholesterol.

Figure 3. Small scale production of algae.

Figure 4. Large scale production of algae.

Figure 5. Twelve-liter fermentor for yeast production.

Figure 6. Two hundred-liter fermentation for yeast production.

<u>Isotope Isomers</u>	
$^{13}\text{CH}_3^{13}\text{COOH}$	81%
$^{13}\text{CH}_3^{12}\text{COOH}$	9%
$^{12}\text{CH}_3^{13}\text{COOH}$	9%
$^{12}\text{CH}_3^{12}\text{COOH}$	1%
<hr/>	
$^{13}\text{CH}_3^{13}\text{COOH}$	63%
$^{13}\text{CH}_3^{12}\text{COOH}$	16%
$^{12}\text{CH}_3^{13}\text{COOH}$	2%
$^{12}\text{CH}_3^{12}\text{COOH}$	3%

Figure 7. Proton resonance spectrum of doubly- ^{13}C labeled acetic acid.

DISCUSSION

Dr. Kipnis: On several occasions I have heard remarks made about carbon-13 glucose and galactose for use in mass screening, particularly in diabetes. I would like to know what the basis is for that experiment.

Dr. Ott: I don't think it has been mentioned for mass screening, but as a clinical trial. It is mainly to demonstrate how carbon-13 could be used in clinical application. If it were to prove useful it would be applicable to general mass screening. This would be fine; but this was really not our primary objective.

Dr. Kipnis: How much material has to be administered?

Dr. Ott: Glucose tolerance studies on 30 patients requires 2.5 kg. On glucose you can get by with about 5% labeled in the C-1 position which requires the synthesis of a couple moles of glucose.

DIAGNOSTIC AND CLINICAL APPLICATIONS OF STABLE ISOTOPES

Chairman: P. D. Klein

VALIDATION AND COMPARISON OF DEUTERIUM/TRITIUM AND CARBON-13/CARBON-14 IN CLINICAL STUDIES OF BILE ACID METABOLISM

*Alan F. Hofmann, Rudy G. Danzinger, Neville E. Hoffmann,
Peter D. Klein, Otto W. Berngruber, and Patricia A. Szczepanik*

USE OF ISOTOPIC TRACERS IN THE STUDY OF GENETIC DISORDERS OF METABOLISM IN CHILDREN

William L. Nyhan

RADIORESPIROMETRY STUDIES OF THE EFFECTS OF L-DOPA AND MK486 THERAPY--A MODEL APPLICATION OF CARBON ISOTOPES IN THE *IN VIVO* EVALUATION OF INTERMEDIARY METABOLISM DURING CLINICAL DRUG TRIALS

J. A. DeGrazia and M. R. MacLaury

ANNOUNCEMENT

Chairman Klein: On behalf of the program planners and also the speakers this morning, we should make a certain disclaimer. Perhaps those of you who are contemplating the use of stable isotopes are intimidated by the title of the session, namely, Clinical Applications, which makes it sound as if it is cut, dried, and fully operational, and if you haven't been doing it you're not with it, because this is certainly not our intention, and I am sure it is not the intent of our speakers.

In fact, almost all of those whom we contacted for this purpose really demurred strongly that they were not in a position to talk about clinical applications; but in fact I think they represent the earliest applications of stable isotopes, and this is why they are here today.

I think you will see recurrent problems in which everyone is interested. Some of them were raised in the preceding session concerning sensitivity and sample requirements. While these papers will not necessarily give final and unequivocal answers to these questions, they begin to bracket the problem and to give some estimate of what is required to make these measurements.

VALIDATION AND COMPARISON OF DEUTERIUM/TRITIUM AND
CARBON-13/CARBON-14 IN CLINICAL STUDIES OF BILE ACID METABOLISM^{*}

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INTRODUCTION

If stable isotopes were available in quantity at low cost, and if rapid, sensitive, precise, and accurate methods were available for their measurement, their application to clinical investigation and diagnostic test would be widespread. At present, the challenge to the clinical investigator is to use the extremely limited supply of stable isotopes in the best possible manner, while the challenge to the analytical chemist is to develop rapid, turn-key methods that can be taken out of the laboratory without loss of precision, accuracy, and sensitivity.

The aims of the studies reported here were 1) to show that determination of an important metabolic parameter could be assessed equally well by stable and radioactive isotopes, and 2) to compare the results obtained from stable isotopes with those obtained by radioactive isotopes. The second point should give information on a) sources of error in analysis by either method, and b) presence of an "isotope effect" *in vivo*. In addition, simultaneous use of the two nuclides permits the analytical methods to be compared in step-by-step detail, and advantages and disadvantages peculiar to one method are more readily detected.

^{*}Supported in part by National Institute of Health Grants AM 6908 and RR585, by grants-in-aid from the Smith, Kline, and French Company and the Mead Johnson Company, and by the U. S. Atomic Energy Commission.

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Two types of studies in man will be reported. In the first, the turnover rate of an endogenous pool of bile acids was determined by a conventional isotope dilution technique, in which the circulating pool is pulse labeled with a tracer dose of isotopic (tritium and deuterium) bile acid (1,2). From the dilution which is calculated to be present at the time of injection, the mass of material present in the patient may be calculated, and from the rate at which the isotope is lost (assessed by decline in specific activity) the fractional turnover rate may be calculated.

In the second group of studies, still in preliminary form, the rate of oxidation by bacteria of the glycine moiety of an N-glycine conjugated bile acid was determined by measuring the appearance of isotopic carbon dioxide in breath at intervals (3).

Although perturbations in the bile acid metabolism have only recently been recognized to be important in the pathogenesis and manifestations of enteric disease (4), the experimental designs reported here are widely used in clinical medicine, and we believe that the principles advanced in this paper have broad applicability to clinical research and laboratory diagnosis.

BILE ACIDS AND LECITHIN. The bile acids are a family of surface active steroid acids made in the liver by saturation, hydroxylation, and side chain oxidation of cholesterol (5). They are excreted from the liver in polymolecular aggregates (micelles) with lecithin. The bile acid-lecithin micelle has an interior of hydrocarbon chains which acts as a solvent for cholesterol, and the bile acid-lecithin micelle is the only excretory route by which cholesterol (which is completely insoluble in water) can enter the intestinal tract from which it can be excreted (6).

In the small intestinal lumen, the lecithin of the micelle exchanges with fatty acid and monoglyceride produced by pancreatic lipolysis of dietary triglyceride; bile acids now serve to transport these lipolytic products up to the mucosal cell, thus greatly increasing the rate of fat absorption (5,7). Most of the bile acids which are secreted into the intestinal tract are reabsorbed by a transport system located in the last part of the small intestine, the terminal ileum (8,9). The bile acids are transported through the ileal cell to the blood, returning to the liver, where they are transported by hepatic cells from blood into bile. This cyclical movement from bile to intestinal lumen to blood to liver and back to bile is called the enterohepatic circulation, and the amount of bile acids which circulate in this manner is essentially constant in health. The conservation of bile acids by the terminal ileum is remarkably efficient, and only a small amount of bile acids escapes and is excreted in stool. Fecal loss is balanced by hepatic synthesis of bile acids from cholesterol.

Lindstedt (1) showed that one can measure the amount of bile acids which circulate by giving a labeled bile acid and determining the specific activity of biliary bile acids on samples of fasting bile obtained daily for several days. Extrapolation of the specific activity decay curve to zero times gives the specific activity which would have been present if instantaneous mixing had occurred and this, divided into the dose of administered radioactivity gives the amount of circulating bile acids, which is referred to as the bile acid pool. The slope of the specific activity decay curve indicates the rate of replacement of the bile acid pool by unlabeled bile acids and this slope multiplied by the pool size gives the daily synthesis (or loss).

Mathematically, the specific activity decay curve is described by the equation:

$$SA_t = SA_{t_0} \times e^{-kt}$$

where k is the fractional turnover rate in days⁻¹. From this one can derive the equation that pool x k = synthesis.

Bile Acid Metabolism in Cholesterol Cholelithiasis. One of the most common diseases of the biliary tract is the formation of solid concretions in the gallbladder, and this disease is called gallstones or cholelithiasis. In most Western countries, gallstones are composed predominantly of cholesterol. Analysis of bile taken directly from the liver at surgery suggests that in patients with gallstones the bile secreted by the liver is supersaturated with cholesterol (10). Since bile supersaturated with cholesterol could arise either from increased cholesterol secretion or decreased bile acid secretion, it became desirable to measure each of these. Measurement of cholesterol secretion by the liver is extremely difficult but the amount of bile acid secreted can be inferred if the size of the bile acid pool is known, since bile acid secretion is equal to the pool size times the number of cycles per day. About two years ago, Vlahcevic and Swell and their colleagues in Richmond reported that the bile acid pool size in patients with cholesterol cholelithiasis was strikingly smaller than that of control subjects (11) and their data have been used as evidence for decreased bile acid secretion in patients with cholesterol cholelithiasis.

Two lines of evidence suggested to us that the measurement of bile acid pool size and turnover rate could be done conveniently with stable isotopes. First, several years ago we had developed an exchange method for preparing 2,2', 4,4'-³H-bile acids and had shown that the label was stable *in vivo* during enterohepatic cycling (12). The method was readily applicable to the preparation of 2,2', 4,4'-³H-bile acids. Secondly, we had developed GC/MS/AVA capability with high precision and sensitivity (13). The fragmentation pattern of unlabeled bile acids gave a m/e peak at 370 with negligible intensity at 374, whereas the tetradeuterio bile acid gave the anticipated fragment at m/e 374 with little background signal at 370. Thus by using the GC/MS/AVA one could readily determine m/e 374/370 ratios in samples and the change in atoms percent excess with time is equivalent to decline in specific activity.

In addition to biological and methodological feasibility, there were also persuasive medical arguments for developing a technique for characterizing bile acid metabolism by using stable isotopes. First, such a technique could be used for population screening. There is fairly good evidence that cholesterol cholelithiasis is genetically determined at least in part (14-16). If a decreased bile acid pool were the cause of gallstones, the ability to measure the bile acid pool size might permit the detection of high-risk individuals, prior to stone formation. Since one of the bile acids, chenodeoxycholic acid, when administered in large doses remedies the defect in bile composition (17,18), the detection of susceptible individuals could conceivably lead to rational and effective prophylaxis. Second, such a technique would permit measurement of bile acid kinetics during pregnancy, which is considered to be related to cholelithiasis. Third, such a technique could be applied to the study of liver disease, especially in children, in which abnormalities of bile acid metabolism are being increasingly recognized (19). Fourth, such a technique would permit simultaneous kinetic measurement of all three bile acids present in human bile. Fifth, such a technique could be applied to characterize bile acid metabolism in children with hyperlipidemic states. Abnormal bile acid metabolism has recently been shown to occur in Type 5 hyperlipidemias. An obvious corollary is that determination of bile acid kinetics in children might permit the detection of latent hyperlipidemic states. Finally, such a technique could prove to be of great value in undergraduate medical education, where students could carry out experiments on themselves safely.

Accordingly, ^2H and ^3H bile acids were prepared as described (12). Women, incapable of childbearing, and with functioning gallbladders (by X ray) containing radiolucent gallstones, were admitted to the Clinical Research Center. A radio-opaque, polyvinyl tube was placed in the third portion of the duodenum by fluoroscopy. Patients received 50 mg ^2H bile acid and 50 μCi ^3H bile acid intravenously on the evening prior to the taking of the first sample. Bile samples were obtained fasting, after intravenous pancreozymin stimulation to cause gallbladder contraction, at days 1,2,3,5, and 7. Samples were deproteinized, saponified, esterified with methanol, separated into dihydroxy and trihydroxy fractions by column chromatography on alumina, and acetylated; details will be published. One aliquot of the methyl ester acetates was used for determination of mass by gas chromatography. A second aliquot was sent to Argonne National Laboratory for determination of Deuterium content by GC/MS/AVA.

Estimates of pool size by the two methods agreed reasonably well (Figure 1). The patients with cholelithiasis have considerably smaller pool sizes of chenodeoxycholic acid and the precision of pool size determination by ^2H labeled bile acids is clearly sufficient to distinguish the two patient groups (Figure 2). Estimates of turnover rate (Figure 3) do not agree nearly as well. The regression line calculated for the points is shown. Since each point represents the slope of a line determined from 4 or 5 time points, it seems quite likely that the slope is significantly greater than 1.00. This would indicate that a slightly greater proportion of the ^2H bile acid is

replaced daily than is the ^3H bile acid. Physiologically, this could be accounted for by a very slightly higher reabsorption of ^3H bile acid than the ^2H form. For example, in six enterohepatic cycles, the difference between 91% and 93% reabsorption would result in a fractional turnover rate of $6(1-0.91)$ or 0.54 versus $6(1-0.93)$ or 0.45. Such a differential is possible if the 3-hydroxy group is involved in active ileal transport, because of the adjacent locations of the hydrogen isotopes. Their influence on binding would be expected to be in the sequence: $^3\text{H} > ^2\text{H} > ^1\text{H}$. Such an effect is also consistent with the isotope effect on the chromatographic mobility of ^3H labeled bile acids (12) and is of the same magnitude. When bile acids labeled in the 26 position with ^{13}C become available, it should be possible to investigate this possibility and determine if such preferential reabsorption takes place.

In the course of these analyses, a number of sources of analytical error were identified and counter-measures developed (Table I). With GC/MS/AVA the specific activity determination is contained in the read-out, whereas with radioactive bile acids, mass and radioactivity must be determined separately. Ideally, we should compare the precision of the two methods by defining analytical error on a standard set of compounds. Such a study is in progress. For ^2H bile acids the only important mass measurement is the material which is administered to the patient.

Our data indicate that the measurement of bile acid pool size and turnover rate by ^2H bile acid is sufficiently precise and accurate to be useful for the detection of disease, as well as characterization of bile acid metabolism in health. Other than the development of analytical capability, the major difficulty associated with the use of ^2H bile acids has been the labor required for synthesis of the 3-keto precursors to be used for exchange labeling, as well as that required for repeated column passages which are necessary to achieve equilibration with the adsorbed $^2\text{H}_2\text{O}$. We are currently exploring the possibility of adding a fifth deuterium atom by reducing the 3-keto compound (after exchange) with sodium borodeuteride.

A Breath Test for Bile Acid Deconjugation Physiology. After being formed in the liver from cholesterol, the bile acids are "conjugated" with either glycine or taurine, i.e., the carboxyl group of the side chain is linked in amide linkage to the nitrogen of the amino acid. These conjugated bile acids may be considered as members of the N-acyl glycine and N-acyl taurine families. Conjugation improves the solubility of bile acids, and since the conjugated bile acids are much stronger acids than the unconjugated acids, they are ionized at the pH of the proximal small intestine, and cannot be absorbed passively into the lipid part of the cell membrane of the jejunum (5,9).

A small fraction escapes the terminal ileum and passes into the bacteria-rich environment of the large intestine. Here, the glycine and taurine are liberated by the bacterial deconjugases or cholyamidases (21,22). It is well known that bacteria rapidly liberate $^{14}\text{CO}_2$

TABLE 1
SOURCES OF ANALYTICAL ERROR WHEN DETERMINING BILE ACID KINETICS
BY ISOTOPE DILUTION

<u>^2H-LABELED BILE ACIDS</u>	
<u>Origin</u>	<u>Countermeasure</u>
^2H bile acid chemical purity	Assay enzymatically with steroid dehydrogenase (mass critical)
^2H bile acid nuclide content	Prepare series dilutions with unlabeled bile acid; obtain standard curve
Background contribution to ^2H bile acid ion intensity	Measure background ion intensity with ^1H bile acid standard

<u>^3H-LABELED BILE ACIDS</u>	
<u>Origin</u>	<u>Countermeasure</u>
^3H counting statistics	Administer adequate activity Obtain sufficient counts
^3H bile radiochemical purity	Prepare chromatographically Assay purity by radiochromatography (mass unimportant)
GLC measurement of unlabeled bile acid mass	Use internal standard Determine detector response for identical mass in comparable mixture

from glycine-1- ^{14}C and that CO_2 diffuses readily across the colonic wall. If so, the rate of appearance of $^{14}\text{CO}_2$ in breath after administration of chylol glycine-1- ^{14}C should be a measure of exposure of the bile acid pool to deconjugating bacteria. The remote possibility exists that some glycine-1- ^{14}C might be absorbed as such, but liver and kidney enzymes are also known to release $^{14}\text{CO}_2$ rapidly from glycine-1- ^{14}C .

Increased bile acid deconjugation should occur in two instances: 1) when bacteria overgrowth in the small intestine occurs or 2) when bile acid malabsorption causes an increased fraction of the bile acid pool to pass into the colon. Validation and results of preliminary application of the test have been published elsewhere (3,23). Some typical results are shown in Figure 4.

The test is based on the assumption that appearance of $^{14}\text{CO}_2$ in breath may be used to infer the rate of a bacterial hydrolysis step, mediated exclusively by bacterial enzymes. This test is to be contrasted to ^{14}C lactose breath test (24), designed for the detection of lactase deficiency, where the appearance of $^{14}\text{CO}_2$ in breath reflects the relative rates of oxidation by tissue enzymes of absorbed lactose versus oxidation by bacterial enzymes of unabsorbed lactose, as well as to tests (25) aimed at detecting disease by changes in the rate of total oxidation of tissue substrate such as glucose.

We wondered then if this test could be performed with a stable isotope. Ingested chylol glycine-1- ^{14}C is diluted by the endogeneous pool of chylol glycine, but in addition, liberated $^{14}\text{CO}_2$ is diluted by the CO_2 pool in the colonic lumen, if such exists, and by the entire body pool of CO_2 . Based on the sensitivity of our instrument, we calculated that about 5 mM of chylol glycine-1- ^{13}C would be required to achieve sufficient atoms percent excess in expired CO_2 for accurate detection.

Since the breath test as described had been carried out using a very small mass of carrier, it was necessary to repeat the breath test using 5 mM of chylol glycine. We measured the appearance of $^{14}\text{CO}_2$ after either 50 mg of chylol glycine-1- ^{14}C or 2,000 mg of chylol glycine-1- ^{14}C and chylol glycine-1- ^{13}C * in three normal subjects and one subject with bile acid malabsorption because of ileal resection. Each test was repeated twice in random order in the four patients.

The results (Figure 5) indicated that, at least in these four subjects, the test was identical with the two doses. Further application of the breath test with ^{13}C will require much greater availability of ^{13}C at low cost.

* Glycine-1- ^{13}C (generously provided by Dr. Donald G. Ott, Los Alamos Scientific Laboratory) was also used in these experiments, but analyses of $^{13}\text{CO}_2$ in breath have not been completed.

Breath testing is extremely simple and rapid, and the clinician will use breath tests commonly when they become available. A very great advantage of breath tests involving stable isotopes is that they can be carried out in patient-care areas by personnel lacking training in radiation safety and without the very expensive insurance required for areas where radiation is used. The pediatrician eagerly awaits the availability of ^{13}C breath tests since collection of expired air from the small child is simple and radiation is not a consideration. Lastly, and only partially in jest, it should be noted that every investigator spends an unseemly number of hours completing elaborate forms for radiation safety committees, which in turn must wrestle with the touchy problems of benefit versus risk in all experiments involving the use of radioisotopes. Every investigator would be delighted to be rid of these very necessary but bothersome safeguards. For these reasons, an exponential increase in the availability of the ^{13}C isotopes is a consummate greatly to be desired.

ACKNOWLEDGMENT

We acknowledge the skillful technical assistance of Miss Jan Carter, Mrs. Linda Moskalik, Mr. Richard Tucker; the aid given in patient studies by the Staff of the Clinical Research Center (Miss Lee Fast, Supervisor); and the skillful secretarial assistance of Miss Doreen Johnson.

REFERENCES

1. Lindstedt, S. The turnover of cholic acid in man. *Acta Physiol. Scand.* 40, 1-9 (1957).
2. Hofmann, A. F., Schoenfield, L. J., Kottke, B. A., and Poley, J. R. Methods for the description of bile acid kinetics in man. In: *Methods in Medical Research*, Yearbook Publishers, Chicago, Illinois, 1970, 149-170.
3. Fromm, H., and Hofmann, A. F. A breath test for altered bile acid metabolism. *Lancet* 2, 621-625 (1971).
4. Hofmann, A. F., and Kern, F., Jr. The significance of bile acids in gastrointestinal and hepatic disease. *Disease-A-Month*, 1971, 1-38.
5. Hofmann, A. F., and Small, D. M. Detergent properties of bile salts: correlation with physiological function. *Ann. Rev. Med.* 18, 433-476 (1967).
6. Carey, M. C., and Small, D. M. The characteristics of mixed micellar solutions with particular reference to bile. *Amer. J. Med.* 49, 590-608 (1970).
7. Hofmann, A. F. The function of bile in the alimentary canal. In: *Handbook of Physiology*, Section on the Alimentary Canal, Vol. V, Sec. 6. Amer. Physiol. Soc., Baltimore, 1968, 2507-2533.
8. Weiner, I. M., and Lake, L. Bile salt absorption: enterohepatic circulation. In: *Handbook of Physiology*, Section on the Alimentary Canal, Vol. III, Sec. 6. Amer. Physiol. Soc., Baltimore, 1968, 1439-1455.
9. Dietschy, J. M. Mechanisms for the intestinal absorption of bile acids. *J. Lipid Res.* 9, 297-309 (1968).
10. Small, D. M. The formation of gallstones. *Adv. Intern. Med.* 16, 243-264 (1970).
11. Vlahcevic, Z. R., Bell, C. C., Jr., Buhac, I., Farrar, J. T., and Swell, L. Diminished bile acid pool size in patients with gallstones. *Gastroenterology* 59, 165-173 (1970).
12. Hofmann, A. F., Szczepanik, P. A., and Klein, P. D. Rapid preparation of tritium labeled bile acids by enolic exchange on basic alumina containing tritiated water. *J. Lipid Res.* 9, 707-713 (1968).
13. Klein, P. D., Haumann, J. R., and Eisler, W. J. A gas chromatograph-mass spectrometer-accelerating voltage alternator for the measurement of stable isotope ratios in organic molecules. *Anal. Chem.* 44, 490 (1972).

14. Danzinger, R. G., Gordon, H., Schoenfield, J., and Thistle, J. L. Lithogenic bile in siblings of young patients with cholelithiasis. *Gastroenterology* 60, 652 (1971). (abstract)
15. Thistle, J. L., Eckhart, K. L., Nensel, R. E., Nobrega, F. T., Poehling, G. G., Reimser, M., and Schoenfield, L. J. Prevalence of gallbladder disease among Chippewa Indians. *Mayo Clin. Proc.* 46, 603-608 (1971).
16. Comess, L. J., Bennett, P. H., and Burch, T. A. Clinical gallbladder disease in Pima Indians: Its high prevalence in contrast to Framingham, Massachusetts. *New England J. Med.* 227, 894-898 (1967).
17. Thistle, J. L., and Schoenfield, L. J. Induced alterations of bile composition in humans with cholelithiasis. *Gastroenterology* 61, 488-496 (1971).
18. Danzinger, R. G., Hofmann, A. F., Schoenfield, L. J., and Thistle, J. L. Dissolution of cholesterol gallstones by chenodeoxycholic acid. *New England J. Med.* In press.
19. Makino, I., Sjoval, J., Norman, A., and Strandvik, B. Excretion of 3 β -hydroxy-5-cholenoic and 3 α -hydroxy-5 α cholanil acid in urine of infants with biliary atresia. *GEBS Letters* 15, 161-164 (1971).
20. Kottke, B. A. Differences in bile acid excretion: Primary hypercholesterolemia compared to combine hypercholesterolemia and hyptetriglyceridemia. *Circulation* 40, 13-20 (1969).
21. Aries, V. and Hill, M. J. Degradation of steroids by intestinal bacteria. I. Deconjugation of bile salts. *Biochim. Biophys. Acta* 202, 526-534 (1970).
22. Midtvedt, T., and Norman, A. Bile acid transformations by microbial strains belonging to genera found in intestinal contents. *Acta Path. et Microbiol. Scand.* 71, 629-638 (1967).
23. Sherr, H. P., Sasaki, Y., Newman, A., Banwell, J. G., Wagner, H. N., Jr., and Hendrix, T. R. Detection of bacterial deconjugation of bile salts by a convenient breath-analysis technique. *New England J. Med.* 285, 656-661 (1971).
24. Sasaki, Y., Iio, M., Kameda, H., Ueda, H., Aoyagi, T., Christopher, N. L., Bayless, T. M., and Wagner, H. N. Jr. Measurement of ¹⁴C-lactose absorption in the diagnosis of lactase deficiency. *J. Lab. Clin. Med.* 76, 824-835 (1970).
25. Shreeve, W. W., Tashjian, A. J., Ogi, N., Slavinski, R. H., and Hoshi, M. Formation of ¹⁴CO₂ and ³HOH from glucose-1-¹⁴C, -1-³H during oral cortisone glucose tolerance tests in obese patients. *Metabolism* 20, 280-292 (1971).

Figure 1. Pool size of bile acid determined by isotope dilution of ^2H bile acids using GC/MS/AVA (ordinate) plotted against pool size determined by isotope dilution of ^3H bile acids using GC/liquid scintillation spectroscopy (abscissa). The equation obtained by fitting these points by the method of least squares gives an intercept of 32.2 ± 41.3 mg (i.e., zero) with a slope of $0.935 \pm .071$ which does not appear to differ from 1.00. The correlation coefficient is 0.983.

Chenodeoxycholic acid pool size in cholesterol cholelithiasis

Figure 2. Pool size, expressed as mg bile acid/kg body weight, of chenodeoxycholic acid, one of two bile acids made from cholesterol in man, in five patients with radiolucent gall stones (left) and six healthy controls (right), matched for age and sex. Although there is clear separation in the values shown here, further analyses (unpublished) have shown some overlap in values for the two groups. The decreased pool size is considered to precede the development of cholelithiasis and to be responsible, at least in part, for the formation of gall stones.

Figure 3. Daily fractional turnover of bile acid determined from slope of specific activity curve of ²H bile acids using GC/MS/AVA (ordinate) plotted against daily fractional turnover rate determined from specific activity decay curve of ³H bile acids using GC/liquid scintillation spectroscopy (abscissa). The equation obtained by a least squares fit of the points gives an intercept of 0.060 ± 0.067 (i.e., zero) and a slope of 1.50 ± 0.26 . The correlation coefficient is 0.922.

SPECIFIC ACTIVITY OF $^{14}\text{CO}_2$ IN BREATH AFTER CHOLYL GLYCINE-1- ^{14}C

(% dose/mole CO_2) $\times$ kg ($M \pm \text{SEM}$)

Figure 4. Time course of $^{14}\text{CO}_2$ excretion in normal patients (solid line) and patients with ileal resection (dotted line) following administration of 20 mg of cholyl glycine-1- ^{14}C . (3)

$^{14}\text{CO}_2$ OUTPUT AFTER 20mg (—○—) or 2000mg (---○---)
CHOLYL GLYCINE-1- ^{14}C

Figure 5. Effect of test loading procedure on production of $^{14}\text{CO}_2$ from cholyl glycine-1- ^{14}C in three normal patients and one patient with bile acid malabsorption. Solid line: 20 mg cholyl-glycine; dotted line: 2000 mg cholyl glycine. In both instances, 10 μC of cholyl glycine-1- ^{14}C was administered.

THE USE OF ISOTOPIC TRACERS IN THE STUDY OF GENETIC DISORDERS OF METABOLISM IN CHILDREN

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ABSTRACT

Isotopic techniques have contributed greatly to the elucidation of disorders of metabolism in man. The metabolic tracer permits the initial study of any newly recognized disease *in vivo*. Most studies have employed ^{14}C labeled compounds. Among the simplest approaches is to assess the oxidation of a labeled compound by determining the isotope content in CO_2 in expired air. More complex studies involve the conversion of a precursor to a known product. In this way, evidence has been obtained in man for the presence of an enzyme, previously recognized only in microorganisms, which converts glycine to CO_2 and to a single carbon compound, ultimately forming the β carbon of serine. Among the most powerful uses of this methodology is the metabolic pattern approach in which methods of generality are employed to monitor the entire spectrum of metabolic products of a labeled precursor. In this way methylcitrate has recently been defined as a metabolic intermediate in man. Methodology to extend these approaches, developed using ^{14}C , to the use of ^{13}C have been initiated. Data on the oxidation of glycine indicate that information can be obtained that is quite comparable to that found using ^{14}C .

We have been engaged for a number of years in the study of inborn errors of human metabolism. Isotopic techniques have been extraordinarily useful in this field in the elucidation of the area and ultimately the site of the enzymatic defect, and also in defining the abnormal chemical environment in which these patients live, which has relevance to the pathogenesis of the clinical manifestations we see. Most of our studies have been carried out using ^{14}C . This provides a background for the applicability of ^{13}C in this field. We have just begun to employ ^{13}C -labeled compounds in the study of metabolism in man.

In a recent issue of *Hospital Practice*, the lead article (1) was the first of a series on nuclear medicine. The author described the many current uses of radioactive materials in clinical medicine and made the point that this is really only the beginning of the use of radioactive materials in man. This may be true, but many of us who

have been in this business for a while have worried increasingly that it may be close to the end. In most institutions it is difficult to obtain approval for the administration of ^{14}C labeled material from committees on human use. In some institutions it is impossible. In the University of California, we have secured approval to employ ^{14}C in children as long as we study patients with metabolic disease. The study of controls is not approved. Patients and parents also have a new attitude about the use of radioactive materials. It is unquestionably much harder to obtain patients for tracer studies now than just a few years ago. These problems and attitudes do not reflect any new information on the hazards of isotopic tracer studies. On the contrary, each year that goes by increases our security that these procedures are safe. The problem is that we are experiencing a new approach to the ecology. No matter how carefully we calculate the radiation dosage, people are concerned about as yet undetermined hazards. In the case of ^{14}C they are concerned about putting something in which is not only radioactive, but is going to be there for 5,000 years. I think there is a real argument for the development of methodology and materials that will permit the productive use of stable isotopes in human biology.

Tracer Techniques in the Study of Purine Metabolism

A major program in our laboratories began with the study of the syndrome that currently bears my name and that of Michael Lesch. We first described this disorder as a new inborn error of metabolism in 1964 (2). Involved patients have a rather severe degree of mental retardation. In addition, they have marked spasticity and other neurological abnormalities which justify a designation of choreoathetosis.

Another feature of the disease is self-mutilation. This is probably the most striking feature of this syndrome. These patients bite their lips, thumbs, or fingers. We have seen two patients who have actually amputated part of a finger by biting. All of the children we have studied with this disease manifest this form of unusual behavior. It catches the imagination in that it is the first example of a clear-cut biochemical disorder which is associated with a stereotyped pattern of abnormal behavior. In this sense it might represent a model for a chemical explanation of behavior.

Some of the other features of the disease illustrate more clearly the fact that we are dealing with a disorder of metabolism. Tophaceous deposits of urate may be seen in the ears or elsewhere. This is a very unusual occurrence in pediatric practice. Uric acid stones may be passed or may cause obstruction or infection. Clinical gouty arthritis may occur quite early in life, at least early in the second decade.

We might consider how one would go about studying this type of metabolic abnormality. The approach we originally employed was the classical one which had been used in the study of adult patients with gout. One may assess the entire sequence of the *de novo* synthesis of purines because of the fact that the glycine molecule is ultimately

incorporated intact into the 4, 5, and 7 positions of the purine ring and consequently found in the uric acid excreted in the urine.

The method involves the administration of carbon-labeled glycine intravenously. Thereafter the urine is collected for 7 days; the uric acid is isolated, crystallized, and its radioactivity determined. Most normal individuals carry on a relatively slow continual process in which about 0.1% of the isotope of glycine that is administered is incorporated into urate in the course of a week. I suspect that what is happening is that the purine formed from glycine goes slowly into the nucleic acids, and as the cells gradually break down, it comes out, ultimately as uric acid.

In patients with Lesch-Nyhan syndrome (Fig. 1) the curves obtained for this process are very different from those of controls (2,3). This tells us quite a lot about the metabolic abnormality. It tells us that there is an enormous overproduction of purine, *de novo*. It clearly distinguishes the patient population from the control population. There are better methods now of making a chemical diagnosis of this condition. However, this overproduction probably remains the best clue as to the pathogenesis of the clinical symptomatology, a problem which remains to be solved.

It had been thought from the time of Hippocrates that abnormalities in purine metabolism did not occur in children. Hippocrates recognized that gout is a disease of the adult. For years uric acid concentrations were not measured in children except in renal failure. It now turns out that there are a number of disorders of purine metabolism that are present in childhood. This type of approach, using isotopic glycine, is one of the ways those disorders can be unraveled.

In the case of the Lesch-Nyhan syndrome the molecular basis of the disease is in the enzyme, hypoxanthine-guanine phosphoribosyl transferase (HGPRT), which in the presence of 5-phosphoribosyl pyrophosphate (PRPP) forms the nucleotides inosinic and guanylic acids from hypoxanthine and guanine. HGPRT can be measured in all cells in the body, including fibroblasts in cell culture and also including erythrocytes. In controls, HGPRT activity is prominent in the erythrocyte. In contrast, in the patients, using conventional methods, we can not find any enzyme at all in the erythrocyte. We do have evidence that we are dealing with a structural gene mutation. The mutant gene determines the synthesis of an HGPRT protein with altered catalytic activity.

Investigation of the Metabolism of Glycine

In order to pursue the metabolism of glycine farther than its involvement in the synthesis of purines, we are led into a new area of clinical presentation, or disease. This brings up the inborn error of metabolism as a cause of overwhelming illness in the newborn period. We have concluded from the study of some patients who present in this way that a large area of unexplored and not yet defined metabolic disease is among those patients who are dying, predominantly

undiagnosed, in the first few days of life. Screening the metabolic disease in the intensive care newborn nurseries has already been rewarding in the discovery of a number of new diseases in recent years. I believe this is going to continue.

Our interest began with the description of a disorder that we thought originally was a primary disorder of glycine metabolism (4). We called it idiopathic hyperglycinemia, because it presented with elevated concentrations of glycine in the blood and other body fluids. A few years later other types of patients were found with hyperglycinemia who had quite different clinical manifestations. Then we broke down the hyperglycinemias into the so-called nonketotic hyperglycinemia and ketotic hyperglycinemia. It was the latter that we had originally described and which has appeared to be the most common. Most of the patients described throughout the world have died of this disease when they were very young, so the number that has been described represents a small part of the total patient population. Involved patients probably have recurrent episodes of ketosis and acidosis, which lead to dehydration and coma, and if not treated very quickly and vigorously, they die. The clinical picture is much like diabetic acidosis, but there is nothing wrong with the blood sugar and there is not anything like insulin available to turn the acute episode off. It is very difficult to treat.

The other group of patients, those with nonketotic hyperglycinemia, is seriously retarded; they have seizures and fail to thrive. All of them are in institutions for the retarded. We believe that nonketotic hyperglycinemia is probably a primary disorder of glycine metabolism. The involved enzyme has not yet been identified, but a considerable amount of evidence points to the location of the defect.

It is now clear that what we have called ketotic hyperglycinemia is a clinical syndrome which can be caused by quite a number of different disorders.

The first of these disorders which can present with the ketotic hyperglycinemia syndrome is now most appropriately called propionyl-CoA carboxylase deficiency. The carboxylase enzyme has been shown to be defective. In addition, there are a number of forms of methylmalonic acidemia, each of which can present with the ketotic hyperglycinemia clinical picture and also with elevated concentrations of glycine in body fluids.

Isovaleric acidemia is another disease that presents with overwhelming illness very early in life. We have recently reported in *Pediatric Research* (5) that it can also present with elevated concentrations of glycine in body fluids.

Figure 2 illustrates an approach to the study of glycine metabolism in man. An isotopic experiment was designed in order to assess the fate of the separate carbons of glycine. It is possible to learn about the metabolism of glycine *in vivo* by using different isotopically labeled

compounds, each labeled on a different carbon. If the label is on the first carbon, the most meaningful study would be that of the CO₂ in the expired air. One could learn something about the decarboxylation of glycine in this way. If you could isolate the serine formed, you would find that it was labeled, because glycine is converted to serine. However, if you degraded the serine using periodate and isolated the β -carbon, there should be no labeling of serine in the 3-position from glycine labeled in the 1-position. The fate of the second carbon of glycine can be traced similarly in the illustration.

The oxidation of glycine has been assessed following the intravenous injection of ¹⁴C labeled glycine. In normal individuals this is very rapid. At the first time point, at about 5 minutes, as early as you can collect a gas sample with a mask and a rubber bag, specific activities are maximal following glycine-1-¹⁴C (Fig. 3). The curve labeled J.E. G-2 indicates that conversion of the second carbon farther to CO₂ is a much slower process. Comparing patient to control we see that T.Z., the patient with nonketotic hyperglycinemia, had a very different pattern of oxidation. He converted carbon 1 of glycine to CO₂ in a slow, leisurely fashion like that of the normal process for carbon 2. We have thought that this might indicate that he may have to involve first a synthetic process before decarboxylation takes place. At any rate, the primary decarboxylation observed in normal individuals is inactive in this disorder.

It is possible in this type of experiment to take blood samples, again at very short intervals--15 minutes, 30 minutes--for by 60 minutes isotope can no longer be found in the blood. Deproteinized samples are then placed in a cation exchange column and the amino acids glycine and serine are isolated. The recovery of isotope in serine has proven that glycine is, in fact, converted to serine in man. The slopes of the curves found in normal individuals were different from those of patients with hyperglycinemia, indicating that the conversion to serine in the patient might be less than perfect. This is true for both ¹⁴C glycines. It was possible to dissect the process further by examining the specific labeling of the third carbon of serine (Fig. 4). We administered glycine which was labeled on the second carbon and isolated the serine from the blood. We degraded the serine with periodate and isolated the β -carbon as the dimedon derivative. The conversion proceeds nicely in control individuals. In contrast, in two patients with nonketotic hyperglycinemia, there was essentially no conversion. The curves representing these patients are actually indistinguishable from background radiation.

This is very close to an assay of an enzyme in an *in vivo* system. There is a defect in something that converts the first carbon of glycine to CO₂ and converts the second carbon of glycine to something that ends up in the third carbon of serine. These data indicate the system that is probably involved. They are consistent with the existence of an enzyme which had not been previously described in man. The enzyme system has been carefully worked out in microorganisms. It is a glycine decarboxylase enzyme system in which the first enzyme

removes the first carbon of the glycine molecule to form CO_2 . The second carbon of glycine ultimately becomes a tetrahydrofolate single carbon derivative which then is a substrate for the synthesis of serine using another molecule of glycine. We believe that these data provide the first evidence that this enzyme system is in fact present in man, and secondly that in patients with nonketotic hyperglycinemia the enzyme is defective.

This experience provides an example of the importance of starting with an *in vivo* experiment. We and others have now tried to pursue this *in vitro*, using fibroblasts. We find the system is not present in fibroblasts and it is not present in erythrocytes. This enzyme system or critical parts of the system must be restricted either to liver or to some other inaccessible organ. If one did not study a disorder like this *in vivo* one would not have any clue as to how to proceed.

Tracer Techniques in the Elucidation of Unusual Products of Metabolism

One of the most powerful uses of tracer techniques to the study of man is to examine the spectrum of products of a labeled precursor. In plants, microorganisms, or experimental animals it is possible to provide the tracer and then subject the entire organism to analysis in order to define the total metabolic pattern. This approach has been very useful in defining biochemical pathways of metabolism. In man we can use a similar approach by administering the tracer and then looking for labeled products of metabolism in the urine. For this purpose we must employ techniques that separate a wide variety of compounds and permit the assessment of the label in places where we do not usually expect a compound. Radioautographic techniques and flow-through counters have been very useful for this purpose. I do not yet know how this type of technique could be applied using stable isotopes. In inborn errors of metabolism in man the identification of interesting, new labeled products of a given precursor may be of special interest for they may give us clues as to the pathogenesis of the clinical manifestations. They of course may also give us insights not previously recognized into normal metabolism.

This approach is illustrated by some of our recent studies on ketotic hyperglycinemia. These particular patients have propionic acidemia. This indicates the presence of a defect in the metabolism of propionate.

I have already indicated my prejudice in a study of any new condition for looking first at metabolism *in vivo*. Therefore, we began to assess the metabolism of propionate by the injection of ^{14}C labeled propionate *in vivo* (8). The type of curves obtained for the conversion of propionate- $1\text{-}^{14}\text{C}$ to $^{14}\text{CO}_2$ were those obtained with glycine- $1\text{-}^{14}\text{C}$. There is very rapid decarboxylation of propionate in normal individuals. Even at the earliest time points the specific activities were maximal. In a patient with methylmalonic acidemia defective metabolism of propionate to $^{14}\text{CO}_2$ was demonstrated. This is consistent with the site of the defect in that disorder in the methylmalonyl-CoA mutase reaction.

In patients with ketotic hyperglycinemia and propionic acidemia there was markedly defective metabolism of propionate. This is one of those problems that can be pursued in the fibroblast. The next step was to show that this same kind of evidence can be obtained in the fibroblast (8). Now it is clear that the defect in these patients is in the propionyl-CoA carboxylase enzyme. This has been demonstrated in cell-free extracts of fibroblasts. The enzyme is essentially inactive in the patient. It is readily measurable in a series of controls. That, I think, is the appropriate pattern of progression from *in vivo* to *in vitro* to enzyme system.

With the defect established, let us return to the experiment in which ^{14}C -labeled propionate was administered to patients and controls. We have examined the organic acids of whole urine collected from the patients for 24 hours following the administration of the labeled propionate. Examination by paper chromatography and radioautography revealed an acid spot that we have never found in normal urine. Furthermore, it was labeled. We pursued this by subjecting whole urine to chromatographic separation on the silicic acid column of an automatic organic acid analyzer. The pattern obtained in control individuals following ^{14}C -labeled propionate involves only a little peak of radioactivity (not very much) in propionate itself. In a patient with ketotic hyperglycinemia, most of the radioactivity was found in a position in which we do not usually see anything, acid or radioactivity. In the patient there was both acid and isotope. Therefore, we had an unknown radioactive compound, a product of propionate not found in normal persons. The area from the organic acid was isolated. Subjected to paper chromatography, it yielded the same spot we had found in the initial paper chromatography of whole urine. The unknown compound also gave a single peak on gas liquid chromatography (GLC).

When we put it through the GLC mass spectrometer, the mass spectrum began to look like something familiar. The pattern looked a lot like that of citric acid, but it was a citric acid plus 14. This was true throughout the spectrum. A molecule resembling citric acid, with an additional mass of 14, would have an additional methyl group. Two possibilities were methylcitrate and homocitrate (Fig. 5). A condensation of acetyl-CoA with α -ketoglutarate would be expected to yield homocitrate. A condensation of propionyl-CoA with oxalacetate, which seemed more likely because the compound was labeled from administered propionate, would yield methylcitrate. Therefore, we obtained authentic methylcitrate and homocitrate; the mass spectrum of the unknown was identical to that of methylcitrate.

We do not know how the compound is made. It would be logical to expect a condensation of the type illustrated. However, this remains to be demonstrated. The existence of this kind of an analog of citrate has very interesting implications for the further pursuit of the pathogenesis of clinical manifestations in these patients. It is also of some interest too as a new metabolite that has not previously been demonstrated in mammalian systems.

Carbon 13 in the Study of Inborn Errors of Metabolism in Man

Through the courtesy of Dr. Donald Ott and with the collaboration of Dr. Peter Klein, we have recently been privileged to undertake an exploration of the metabolism of ^{13}C labeled glycine that you heard about earlier in Don's presentation.

Our initial efforts have been devoted to an exploration of whether or not it is feasible to carry out the kind of experiment in man that we have been discussing so far, using the ^{13}C -labeled compound. Our first experiment was in a hyperuricemic patient with the Lesch-Nyhan syndrome and deficiency of HGPRT. In addition, we have had the opportunity to study another patient with a hyperuricemic disorder in which the HGPRT enzyme is completely normal. We are pursuing the study of purine metabolism in these patients. We are also isolating the glycine and the serine from the blood to assess the applicability of this technique. We can now report on the oxidative metabolism of glycine.

The experiment was designed to study simultaneously the metabolism of ^{14}C -labeled glycine and ^{13}C -labeled glycine. In this way we could determine whether or not the information obtained using the stable isotope was comparable to that obtained with the radioactive one. We injected glycine-1,2- ^{13}C (90% atom excess in a dose of 50 mg/kg or 635 mg) and at the same time glycine-1- ^{14}C (7165 mCi/mM) in our usual dose (2 $\mu\text{Ci/kg}$ or 25.4 μCi). We collected expired air for 2 to 4 minute periods at a number of intervals after injection in 13 liter rubber breathing bags. The CO_2 from the air was trapped in 3 *N* NaOH by evacuating each bag with a pump through a trapping system.

The content of $^{14}\text{CO}_2$ and its specific activity were determined in La Jolla, and the curves were identical to those obtained previously in control individuals. The content of ^{13}C and ^{12}C was determined at Argonne. The data are shown in Table 1. The experiment tells us a number of things. In the first place it tells us, most importantly, that this type of experiment is feasible. We can get meaningful numbers using the ^{13}C . It also tells us that we could do the experiment in terms of the conversion of CO_2 collection with about ten times less labeled material than we used.

The ratios of $^{13}\text{CO}_2$ to $^{14}\text{CO}_2$ have been plotted in Figure 6. Time is represented logarithmically on the abscissa. The relationship was nicely linear. This is also a check on the reliability of the data and the comparability of the information from ^{13}C and ^{14}C . The regression analysis of the line obtained has been analyzed (Table 2). This analysis gives us some interesting information. It is consistent with the information we have obtained in other ways. The curves should represent the normal metabolism of glycine. The patient does have a defect in the conversion of glycine to purine, but that is at maximum about 2% of his total glycine metabolized. There is no reason to believe that this would affect the overall metabolism of the amino acid. Furthermore, the $^{14}\text{CO}_2$ data were identical to what we

have observed in controls. Therefore, the data should represent the normal metabolism of glycine in a child of about 15 kg.

If one calculates from the line the intercept and the slope, the slope is a measure of the relative rates of conversion of CO_2 of the first and second carbon versus the first carbon alone. This² is because the tracers we employed were ^{14}C -labeled glycine which was labeled in the first carbon, and ^{13}C -labeled glycine which was labeled in both carbons. Running through the calculations, K_2 over K_1 turns out to be about 6, indicating that carbon 1 is metabolized to CO_2 about six times as fast as carbon 2. I believe that this is what one would obtain if one did the experiment with two different injections, each with a glycine labeled on a different carbon.

These data indicate that we can extend this methodology. The demonstration that the conversion of glycine to CO_2 can readily be assessed using the stable isotope opens up the field of inborn errors of metabolism to exploration using ^{13}C .

REFERENCES

1. Winchell, H. S. Radioactive Tracers in Medicine. *Hosp. Practice* 6, 49 (1971).
2. Lesch, M., and Nyhan, W. L. A Familial Disorder of Uric Acid Metabolism and Central Nervous System Function. *Am. J. Med.* 36, 561, (1964).
3. Nyhan, W. L. Discussion of Additional Features. In: Seminars on the Lesch-Nyhan Syndrome, edited by J. H. Bland, *Fed. Proc.* 27, 1083, (1968).
4. Childs, B., Nyhan, W. L., Borden, M., Bard, L., and Cook, R. E. Idiopathic Hyperglycinemia and Hyperglycinuria, A New Disorder Of Amino Acid Metabolism. *Pediatrics* 27, 522 (1961).
5. Ando, T., Klingberg, W. G., Ward, A. N., Rasmussen, K., and Nyhan, W. L. Isovaleric Acidemia Presenting with Altered Metabolism of Glycine. *Pediat. Res.* 5, 478, (1971).
6. Nyhan, W. L., Ando, T., and Gerritsen, T. Hyperglycinemia. In: Amino Acid Metabolism and Genetic Variation, Edited by W. L. Nyhan, McGraw-Hill Book Co., New York, 255 (1967).
7. Ando, T., Nyhan, W. L., Gerritsen, T., Gong, L., Heiner, D. C., and Bray, P. F. Metabolism of Glycine in the Nonketotic Form of Hyperglycinemia. *Pediat. Res.* 2, 254, (1968).
8. Ando, T., Nyhan, W. L., Connor, J. D., Rasmussen, K., Donnell, G., Barnes, N. D., Cottom, D., and Hull, D. Oxidation of Glycine and Propionic Acid in Propionic Acidemia with Ketotic Hyperglycinemia. *Pediat. Res.* (In press).

TABLE 1
ISOTOPIC DATA

Time, min.	$^{14}\text{CO}_2$	$^{13}\text{CO}_2$	Ratio
	10^3 dpm	δ	
5' 40"	305.1	328.7 ± 0.5	0.928
14' 10"	344.2	427.6 ± 1.9	0.805
30' 30"	432.2	941.0 ± 1.7	0.459
60'	259.3	438.1 ± 1.4	0.592
120'	156.4	336.3 ± 2.4	0.465
240'	56.2	150.0 ± 3.1	0.374
1440'	2.6	29.0 ± 1.6	0.090

$$\delta^{13}\text{CO}_2 = \left[\frac{^{13}\text{CO}_2/^{12}\text{CO}_2 \text{ sample}}{^{13}\text{CO}_2/^{12}\text{CO}_2 \text{ reference}} - 1 \right] \times 1000$$

TABLE 2
ANALYSIS OF ISOTOPIC DATA

Regression analysis of $\text{dpm } ^{14}\text{CO}_2 / \delta ^{13}\text{CO}_2$ vs Ln time

Intercept: 1.13 ± 0.09

Slope: -0.143 ± 0.002

Slope is a measure of relative rates of conversion to CO_2 of carbons 1 and 2, vs carbon 1 alone; i.e.

$$\text{Slope} = K_1 / (K_1 + K_2)$$

Rearranging

$$K_2/K_1 + 1 = 1/\text{Slope}$$

$$= 6.97$$

$$K_2/K_1 = 5.97$$

Figure 1. Incorporation of glycine- $\text{U-}^{14}\text{C}$ into urinary urate. A dose of $2 \mu\text{Ci/kg}$ was injected intravenously. The four patients are designated by initials. The data represent the cumulative percent of the isotope of glycine administered that was recovered in the uric acid crystallized from the urine. Reprinted with permission from the *Federation Proceedings* (3).

Figure 2. Design of an experiment to assess the metabolism of the separate carbons of glycine *in vivo*. The labeling patterns are those one would expect following the injection of glycine-1- ^{14}C or glycine-2- ^{14}C . C* represents a ^{14}C atom.

Figure 3. The decarboxylation of glycine- ^{14}C to $^{14}\text{CO}_2$ *in vivo*. The data are the specific activities of expired CO_2 . J.E. was a control individual, and T.Z. a patient with nonketotic hyperglycinemia. G-1 indicates an experiment with glycine-1- ^{14}C , and G-2, glycine-2- ^{14}C . Reprinted with permission from McGraw Hill Book Co. (6)

Figure 4. Conversion of carbon 2 of glycine to carbon 3 of serine. M.S. and J.E. were controls; S.F. and T.Z. were patients with nonketotic hyperglycinemia. FAM refers to the formaldemethone derivative of the formaldehyde derived from carbon 3 by periodate oxidation. +G indicates the creation of a large pool of glycine, comparable to that of the patients, by the infusion of nonisotopic glycine in the controls. Reprinted with permission from *Pediatric Research* (Karger, Basel/Munchen/New York). (7)

FORMATION OF METHYLCITRATE

Figure 5. The structures of methylcitrate and homocitrate. Also illustrated is the probable condensation from propionyl-CoA.

Contribution of ^{14}C -Glycine and ^{13}C -Glycine to Respiratory CO_2

Figure 6. Isotopic data. The ratios of the ^{14}C isotope content to the ^{13}C abundance have been plotted against the logarithm of time in minutes.

RADIORESPIROMETRY STUDIES OF THE EFFECTS OF L-DOPA AND MK486
THERAPY--A MODEL APPLICATION OF CARBON ISOTOPES TO THE *IN VIVO*
EVALUATION OF INTERMEDIARY METABOLISM DURING CLINICAL DRUG TRIALS

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ABSTRACT

The rate of turnover of $^{14}\text{CO}_2$ in expired air was measured after the intravenous administration of a number of radiocarbon-labeled compounds that are known to be rapidly decarboxylated in human intermediary metabolism. These studies indicate a possible use of this technique (Radiorespirometry) for the evaluation of drug effects upon human intermediary metabolism. Model studies in patients receiving MK486 or pharmacologic amounts of L-dopa were performed to test this possibility. Results show that treatment with MK486 is associated with a striking dose-dependent reduction of $^{14}\text{CO}_2$ production from $1\text{-}^{14}\text{C}$ L-dopa. Measurements of the radiocarbon and L-dopa excretion in urine indicate that this test is an objective measure of MK486 effect upon the metabolism of L-dopa to dopamine. Although additional studies show that the radiorespirometry test is sensitive to a number of factors affecting the turnover of radiocarbon in metabolic pools, the therapy with MK486 did not significantly alter the $^{14}\text{CO}_2$ expiration patterns obtained with $1\text{-}^{14}\text{C}$ L-tyrosine or when intermediates of the TCA cycle were administered.

It is therefore concluded that this technique can be developed into a simple clinical test in which carbon isotopes can be used for the *in vivo* evaluation of the biochemical response to drug therapy. Tests of this type could significantly increase the information gained in drug testing and at the same time reduce the cost and time required for the completion of clinical trials.

* Supported in part by James Picker Foundation National Academy of Science Grant, U.S. Public Health Service Grant MK17798-01, Stanford University Grant FR-05353-07, and by a gift from Merck, Sharp and Dohme Research Corporation, West Point, Pennsylvania.

Studies were carried out in part on the General Clinical Research Center, Division of Research Resources, RR70.

The physical characteristics of carbon have given this element a fundamental role in human chemistry. In fact, there are few, if any, metabolic processes in which carbon compounds do not participate. Consequently, it is reasonable to expect valuable information about the rates of biochemical reactions in humans from *in vivo* studies of the metabolism of compounds labeled with isotopes of this element. Over the past several years, we have performed a number of pilot studies in which we have tested the possibility that a simple, clinically useful, approach to this problem can be developed. This work has focused initially upon the measurement of relative rates of oxidation of ^{14}C compounds to CO_2 using instruments that automatically monitor the $^{12}\text{CO}_2$ and $^{14}\text{CO}_2$ expired in a patient's breath. Carbon dioxide is a universal metabolic end product and the monitoring of isotopic carbon expired in the breath could be the simplest and most direct approach to test the use of compounds labeled with carbon isotopes in medical diagnosis (1). In this effort, we have performed a number of studies in which we have administered specifically-labeled ^{14}C compounds to patients suffering diseases that are associated with a biochemical alteration that can be documented by other laboratory tests and that are readily returned to normal following therapy (2,3). These have had three basic objectives. They are: 1) to obtain a clinical diagnosis by the identification of biochemical abnormalities that are characteristic of specific diseases, 2) to evaluate drug therapy by providing biochemical evidence for a therapeutic response or a toxic reaction, and 3) to recognize biochemical abnormalities that may contribute to the pathogenesis or progress of a disease before that disease is clinically evident.

Whenever possible, we have shown that the administered compounds do enter the expected pathway, and that there is a good correlation between the radiorespirometry result and the measured accumulation in blood or urine of metabolites from the pathway in question (3-5). We conclude from these and similar studies by others that tests of this type can be a very powerful tool for the *in vivo* investigation of human intermediary metabolism (6-14). In this paper, I would like to show some of the results that we have obtained which we believe support these conclusions. First, a brief word about the method used.

All of our studies have been done using ^{14}C isotope. However, as you will see, this approach provides an excellent model system for the development of techniques using compounds labeled with other carbon isotopes. Figure 1 shows the equipment we have in our division for these studies. Our subjects are usually hospitalized in the controlled environment of the Stanford Hospital Clinical Research Center. Here, they are placed on a controlled dietary intake. On the morning of the test, the patient is placed in the clear plastic helmet of the metabolic gas analyser and appropriate baseline data are obtained. From 10-15 μCi of a radiocarbon-labeled compound with specific activity of approximately 2-10 mCi/mM are then administered intravenously.* Expired air then flows through the system at constant temperature and pressure

* Isotopes used were obtained from Amersham Searle, New England Nuclear or Calbiochem Corporations.

to the infrared CO₂ analyzer and to the ionization chamber with attached vibrating reed electrometer. The ¹²CO₂ and ¹⁴CO₂ content of expired air is thus monitored continuously. Our instrument signals may be displayed on an analogue chart recorder or a digital printer. In addition, we have an interface that will transmit this information to an IBM 1800/360-50 computer. At the completion of these studies, the data is returned from the computer to our teletype terminal, and x/y plotter, or to the CRT graphics display shown in Figure 2. The technique used to analyze the data obtained with this system is graphically illustrated in Figure 3. In this case, approximately 20 µCi of 1-¹⁴C-L-dopa was administered to a control subject. The data points are the ¹⁴C/¹²C breath activity measured at one minute intervals, and normalized for the patient's body weight and dose of isotope administered. We also calculated the fractional excretion of radioactivity for ½, 1, and 2 hours, and the time at which the maximum breath activity is reached (T_{max}).

The ¹⁴CO₂ expiration curve that is superimposed upon the data points was obtained by an iterative least squares curve-fitting procedure, using an equation containing the sum of three exponents. Figure 4 compares the radiorespirometry curves obtained in this manner when five different amino acids labeled with ¹⁴C were administered to a control subject. Some of these curves are very similar--for example, there are only slight differences between the curves obtained with 3-¹⁴C serine and 1-¹⁴C L-tyrosine. On the other hand, the data obtained when 2-ring-¹⁴C histidine is given are quite different. Thus, the ¹⁴CO₂ expiration pattern is often (but not always) different when different compounds are given. The radiorespirometry studies will also reflect the location of the labeled carbon in the same compound. For example, the uppermost curve of Figure 5 was obtained when 1,4-¹⁴C succinate was administered to a control patient. The lower curve was obtained when 2,3-¹⁴C succinate was administered to the same patient. I believe that a great deal of useful information about *in vivo* metabolism can be obtained from observations such as these. I would like to review with you data that we have obtained from studies of L-dopa metabolism that illustrate how this is possible. These studies fall into the second group of objectives outlined before. That is, they are a model for the development of clinical tests using carbon isotopes for the *in vivo* evaluation of drug effects upon human chemistry.

In general, drug effects upon oxidative metabolism can be detected by radiorespirometry techniques if 1) there is a specific block in the further metabolism of the compound to be tested, or 2) if there is a non-specific change in the size or rate of turnover of the pools entered by the labeled intermediate. I will discuss these two effects separately. The first will be illustrated by results of studies of the effect of MK486 upon L-dopa metabolism to dopamine. The second will be illustrated by the results of studies with L-dopa precursors tyrosine and phenylalanine and intermediates of the TCA cycle during L-dopa or phenylalanine loading.

The Effect of the Decarboxylase Inhibitor,
MK486, on L-dopa Metabolism

Low dopamine levels and reduced dopamine decarboxylase activity has been reported in the brain striatum of patients with Parkinson's disease. Thus, altered metabolism of L-dopa to dopamine appears to be an important biochemical defect in this illness (15,16). Currently, treatment of Parkinson's disease attempts to compensate for this defect by the use of drugs which will elevate brain dopamine levels. Unfortunately, the administration of dopamine itself will not do this since this amino acid is not able to cross the blood/brain barrier. However, L-dopa, the immediate precursor of dopamine, readily enters brain tissue, and is then promptly metabolized to dopamine. Striking clinical improvement has been reported in patients receiving therapy with this agent (17,18). On the other hand, a significant fraction of Parkinsonian patients receiving L-dopa do not show significant clinical improvement. This is presumably due to the rapid metabolism of dopa to dopamine in other areas of the body. Consequently, dopa brain levels are not sufficiently elevated and the increased peripheral metabolism of L-dopa produces a significant incidence of unwanted side effects (19,20). However, the rather non-specific enzyme required for the further metabolism of L-dopa can be selectively blocked in all tissues except brain by the administration of the L-dopa analogue L- α -methyldopahydrazine (MK486). The addition of this drug to therapy with L-dopa should reduce toxic side effects by lowering peripheral dopamine levels and at the same time, potentiate the beneficial effects of administered L-dopa by raising L-dopa levels in the blood, and therefore, the brain (21-24). The reactions affected by this treatment are outlined in Figure 6. Carbon dioxide is a product of the metabolism of L-dopa to dopamine, and since it is cleared from the body mainly via the lungs, increased doses of decarboxylase enzyme inhibitor should cause a progressive reduction in the amount of CO_2 appearing in the expired air from L-dopa (24). The measurement of this change could be used to determine the amount of drug needed for effective therapy.

We have tested this hypothesis in 25 studies of six patients with Parkinson's disease and three control subjects who are participating in a clinical trial of the beneficial effects of therapy with L-dopa and/or MK486.* These individuals were given from 15 to 25 μCi of ^{14}C -L-dopa as a single intravenous bolus. This was given in the morning, following an overnight fast, and one hour after the administration of appropriate drug therapy. Figure 7 shows the changing $^{14}\text{CO}_2$ expiration pattern that was obtained in these studies when varied doses of MK486 were given to the same patient. There was a striking reduction in the breath excretion of $^{14}\text{CO}_2$, which is greatest when the dose of MK486 was increased from 25 to 200 mg per day. Also of interest is the fact that the 200 mg dose was relatively less effective than the 25 mg dose. Additional studies (Figure 8) confirm our suspicion that the effect of

*We are grateful to Merck, Sharpe and Dohme Corporation, West Point, Pennsylvania, for supplying the MK486 (α -methyldopahydrazine) used in these studies.

MK486 was maximal at approximately 50 mg per day, and increasing doses of 100 and 200 mg per day have little further effect. Table I summarizes the results of all our studies in this series. The control groups consisted of untreated normal subjects and patients receiving a daily dose of 2.5 g of 5-hydroxytryptophane or from 4 to 7 g of L-dopa. No significant differences are noted between the results obtained in these groups of studies.

On the other hand, a dose-dependent reduction in the amount of $^{14}\text{CO}_2$ recovered in expired air was noted in all tests in which MK486 was administered. Since oral loading with L-dopa did not change the radiorespirometry result, it is reasonable to conclude that ^{13}C -labeled compounds could be used in these studies. Actually, 1-2 g of L-dopa is routinely administered orally in divided doses of 250 or 500 mg per day to patients receiving MK486 therapy. If we assume prompt and efficient absorption of this isotope (100%), we would expect to recover approximately 1 mg of carbon as $^{13}\text{CO}_2$ in the first 30 minutes of our test. At the same time, approximately 80 mg of ^{13}C would be produced by ordinary metabolism, assuming that expired air has a 4% CO_2 content and is exchanged at 12-15 liters per minute. In this case, a method that could detect this isotope in expired air must have a precision that will permit the detection of the addition of 1 mg ^{13}C to a carbon pool containing 80 mg ^{13}C and 8 g of ^{12}C as CO_2 . I am told that this precision is quite reasonable for a high resolution ratio mass spectrometer. For example, the Atlas instrument at Stanford University has a sensitivity of 1 o/o when operating under routine conditions. With care, it is possible to increase this sensitivity by a factor of three or better. However, cautious interpretation of these results is warranted since other compounds may not be so rapidly metabolized or so well tolerated as L-dopa. I think it is reasonable to predict from these results that instruments will have to have precision sufficient to measure .1 to 1 mg added to 1000 mg of ^{12}C if the broad clinical potential of studies such as these is to be fully realized.

There may be some concern that the administration of drugs such as MK486 will result in increased retention of isotopic carbon. This was not the case in these studies. The data in Figure 9 shows that total radiocarbon excretion in urine and breath actually increased slightly as the MK486 dose was increased to 100 mg daily and then it fell as the amount of drug given was further increased to 200 mg. The cause for this is not clear from these studies, but it is possible that clearance of metabolites is reduced as the smallest dose of drug giving peak effect is exceeded. This effect was reversed by oral L-dopa loading and the administration of 2 g of L-dopa resulted in nearly complete recovery of administered isotope. Thus, if isotope retention is used as the criterion, the hazard due to isotope effects in loading studies with either ^{13}C or ^{14}C L-dopa is minimal.

Additional studies were performed to better define the specificity of the radiorespirometry test. For this it was necessary to measure L-dopa levels or those of related metabolites present in blood or

urine at various times following the initiation of the study. Plasma levels of L-dopa were not measured in these studies, but they are known to be slightly elevated by combined therapy with MK486 and L-dopa (22). We did, however, measure a striking elevation in urinary L-dopa excretion when L-dopa + MK486 was given (25,26). This was in marked contrast to the slight reduction of L-dopa excretion that was observed when only MK486 was given. These results appear in Figure 10.

In addition, the $1\text{-}^{14}\text{C}$ L-dopa was consistently a small fraction (approximately 20%) of the total ^{14}C appearing in the urine (Figure 11). This relationship did not change significantly, since both total radiocarbon excretion and the excretion of $1\text{-}^{14}\text{C}$ L-dopa rose as the MK486 dose was increased. As seen in Figure 12, these changes in L-dopa and ^{14}C L-dopa excretion resulted in an increase in urinary L-dopa specific activity as the MK486 dose was increased to 100 mg daily. Urinary specific activity then fell when 200 mg per day was given and was lowest when 2 g of L-dopa was added to this regimen, indicating expansion of the L-dopa pool. A comparison of these results to the radiorespirometry data of Table I shows that the increased urinary specific activity at low MK486 doses was associated with a fall in breath specific activity, while expansion of the L-dopa pool by increased doses of MK486 or L-dopa loading did not significantly change the radiorespirometry results.

From these data, we conclude that the radiorespirometry test is an objective measure of the blocking effect of MK486 upon L-dopa decarboxylase. Assuming that the beneficial effect of the drug is derived only from the increased metabolism of dopa to dopamine in the brain (as initially postulated), we would predict that the maximum dose administered to these patients should not exceed 100 mg daily. These results correlate well with clinical trials that indicate good patient response at this dose and that toxicity may occur at levels exceeding 100 mg daily. Further, the relatively low fraction of total radiocarbon appearing in the urine as L-dopa appears to confirm the suggestion that metabolism of L-dopa occurs by alternate pathways which do not involve decarboxylation.

Several other minor pathways for L-dopa metabolism have been described (27,28) and these are summarized in Figure 13. For example, the further metabolism of L-dopa to melanin precursors, 3-hydroxy-4-methoxy-L-dopa or conjugates of L-dopa would not involve decarboxylation and removal of radiocarbon label as CO_2 . Compounds such as these will be excreted in the larger non-dopa fraction of isotope in the urine along with metabolites containing recycled radiocarbon. It is also possible that alternate pathways such as the decarboxylation of 3,4 dihydroxyphenylpyruvic acid contributes to the ^{14}C appearing in the expired air. This would be at least a partial explanation for the failure of MK486 to completely block L-dopa decarboxylation. However, animal studies indicate that MK486 may also inhibit other decarboxylation reactions, including the one shown on this figure. If this is true, then CNS metabolism may contribute greatly to the $^{14}\text{CO}_2$ appearing in the breath when maximal doses of MK486 are given.

Related Studies of TCA Cycle Metabolism, Tyrosine, and Phenylalanine

Figure 13 also shows that the L-dopa precursors phenylalanine and tyrosine can be alternatively metabolized to CO_2 via the TCA cycle. Since there is no requirement for L-dopa decarboxylase in this series of reactions, they should not be directly affected by MK486 treatment. On the other hand, there are a number of ways that drugs can ultimately alter the radiorespirometry results. For example, they may alter isotope distribution by changing blood flow to specific organs, modify transport mechanisms into cells or induce changes in the size of metabolite pools that supply or are supplied by a metabolite pathway that has been specifically altered by drug therapy. Our studies of the effect of L-dopa loading and MK486 therapy upon this alternate pathway showed that radiorespirometry tests are sensitive to at least some of these factors. I would now like to consider the evidence that we have accumulated as we have developed the L-dopa model that supports this conclusion.

A. The Importance of Metabolite Pooling

The $^{14}\text{CO}_2$ expiration patterns obtained when four different isotopes of acetate and succinate were given to a control subject are compared in Figure 14. Each curve is different. Since the isotopes used differ only in the location of the radiocarbon label on the same compound, the different results must reflect some aspect of intracellular metabolism. Table II shows that this result was easily reproduced in five studies of control subjects receiving 1,4- ^{14}C succinate and 2,3- ^{14}C succinate, and that the differences within each group of studies are small and there is no overlap between the two groups. For example, the T_{max} varied from 5 to 6 minutes for 1,4- ^{14}C succinate and from 20 to 30 minutes for 2,3- ^{14}C succinate.

The reasons for the differences observed with these compounds will be understood if we take a moment to refresh our memories about the chemistry of the citric acid cycle (29). This is outlined in Figure 15. A process of continued recycling and redistribution of carbon from one metabolite to another within this pathway is required until that carbon is completely removed by decarboxylation of oxalosuccinate to α -keto-glutarate. The labeled carbon of each intermediate used in these studies has a different path within the TCA cycle. The location of the labeled carbon upon the entering metabolite will determine its rate of turnover within the system. Of the compounds used in these studies, we would expect the rate of release to be fastest for 1,4- ^{14}C succinate, since this label is removed completely on one pass through the system. It will be progressively slower for 5- ^{14}C - α -keto-glutarate, 1- ^{14}C acetate, 2,3- ^{14}C succinate and 2- ^{14}C acetate in this order, since the degree of recycling increases for each of these compounds in this order. The differences in the turnover of isotopes are accentuated by the continuous influx of unlabeled carbon from ordinary metabolism and by the loss of isotope from the TCA cycle through exchange or synthesis. The overall result of these processes will be a reduction of the $^{14}\text{C}/^{12}\text{C}$ breath specific activity and a

delay in the rate of appearance of radiocarbon in expired air. Consequently, a different radiorespirometry result would be expected with each differently labeled pair of acetate or succinate compounds (30,31). This conclusion is also supported by comparative studies of propionate and succinate oxidation. Figure 16 shows that 1-¹⁴C propionate and 2-¹⁴C propionate must first be metabolized to 1,4-¹⁴C succinate and 2,3-¹⁴C succinate respectively before they are completely oxidized to CO₂ (6,32,33). Thus, it should be possible to assess the relative effect of metabolism occurring within and without the TCA cycle upon the radiorespirometry pattern by a comparison of the results obtained in studies with each pair of compounds.

Figure 17 shows that when this was done, the results obtained in tests with 1-¹⁴C propionate and 2-¹⁴C propionate are nearly identical to those obtained with 1,4-¹⁴C succinate and 2,3-¹⁴C succinate, respectively. It appears, therefore, that the radiorespirometry pattern for these compounds is determined primarily by events occurring within the TCA cycle. These are clearly more important than factors common only to each pair of compounds, such as distribution or transport into or the release of CO₂ from metabolite pools. Thus, our original hypothesis is confirmed; that is, a) our test is sensitive to metabolite pooling within the system tested and b) pooling within this system is the major determinant of the ¹⁴CO₂ expiration pattern obtained from labeled metabolites entering the pathway.

Additional studies indicate that the radiorespirometry test may also be sensitive to relative differences in the rate at which isotope enters the TCA cycle. For example, Table IV compares the results of studies with 5-¹⁴C-α-ketoglutarate to the data obtained when all four labeled acetate or succinate compounds were administered to control subjects and patients receiving L-dopa therapy. Since 5-¹⁴C-α-ketoglutarate is the immediate precursor to 1,4-¹⁴C succinate, we might have expected the same degree of identity between the studies with these two compounds that was observed in our comparison of the propionate and succinate studies. Instead, the turnover of radiocarbon from α-ketoglutarate is relatively slow. This delay can be explained by the existence of a relatively large α-ketoglutarate pool, by a reversible exchange of label between other pools such as glutamine or by the existences of a difference in the rate of transport of α-ketoglutarate within the cell to the metabolizing site. Although a relative barrier to α-ketoglutarate penetration of mitochondria has been described (34, 35), our test is non-specific and it is obvious that we cannot say which of these is most important from our studies. However, additional insight into the possible cause for this result could be gained from further studies with related compounds in the future.

B. The Effect of Oral L-Dopa Loading and MK486 Therapy

The same general pattern was seen for all studies of control and L-dopa treated subjects (Table IV). The only significant difference between these two groups is the generally lower specific activity in all

studies in the Parkinson's group. This is a rather non-specific finding that is seen in the results of all studies in this series and thus most likely it is due to technical factors* or to a general alteration in metabolism that may be related, for example, to the debility of the Parkinson's population rather than an effect of L-dopa therapy. Thus, it appears that the rates of decarboxylation of metabolites of the TCA cycle were not altered during this therapy.

No differences were noted between the radiorespirometry results obtained when 1,4- ^{14}C succinate and 2,3- ^{14}C succinate was administered to Parkinson's patients before and after treatment with MK486. Only a slight difference of uncertain significance was observed in a similar test with 1- ^{14}C -L-tyrosine. When these results are compared to those obtained after the administration of 1- ^{14}C -L-dopa to the same patient, it is clear that the MK486 effect is limited mainly to the oxidative decarboxylation of L-dopa. Thus it is reasonable to conclude that although our studies show the radiorespirometry test is affected by a number of factors, including direct drug effects upon specific enzyme systems or changes in the size or rate of turnover of metabolite pooling, there is no evidence in our series of studies to suggest that MK486 directly or indirectly affects the turnover of radiocarbon in this important alternate pathway for the metabolism of L-dopa precursors.

C. The Effect of Metabolite Loading

Finally, I would like to comment briefly upon the importance of the load of metabolite that is used in the experimental design of the radiorespirometry tests. In general, tests with ^{13}C will more closely parallel the results obtained in isotope studies with very low specific activity materials. It is important to realize that as the specific activity of the isotope is reduced, the radiorespirometry test becomes a measure of the patient's response to a biochemical stress rather than a measure of the unperturbed rate of metabolism of the administered compound. This is illustrated in Figure 18 by the changing radiorespirometry pattern that was observed when the amount of carrier phenylalanine administered with 1- ^{14}C -L-phenylalanine was varied in studies of control subjects. There is a nearly five-fold increase in the breath specific activity as the amount of phenylalanine was increased to 30 mg/kg. However, the patient could not respond to further

*Most of the control studies in this series were performed while the author was at the San Francisco General Hospital. Thus, the patient studies are not simultaneous with the control studies. Since the two instruments have different performance characteristics, it is possible that differences in their calibration could account for the apparent reduction in total ^{14}C production observed in the patient group.

loading beyond this dose and the breath specific activity then fell to lower values in subsequent studies. The ability to respond to a challenge of this type may be limited in individuals who have or are prone to having a specific disease. Tolerance tests of this type may be very helpful in detecting persons with latent or hidden metabolic error. For example, the measurement of blood phenylalanine levels after loading with phenylalanine has been proposed as a screening test for the detection of carriers of the trait for phenylketonuria (36,37). Another more commonly used example is the use of glucose loading in a tolerance test to distinguish the diabetic from the non-diabetic state (38). Clearly, radiorespirometry studies using ^{13}C labeled compounds would be well suited for tests of this type.

CONCLUSION

Our results indicate that respirometry studies using carbon isotopes can provide a clinically useful approach to the *in vivo* investigation of drug effects upon human intermediary metabolism. Since this information is often obtained in advance of the full development of clinical signs indicating drug toxicity or response, tests of this type should significantly reduce the hazard of drug overdosage, lessen the cost and speed the completion of clinical drug trails. There are three isotopes of carbon, (^{11}C , ^{14}C , and ^{13}C), that can be used for this purpose. Each has its own special advantage and limitation for use in studies of the type that I have described. Of these, only ^{13}C is not radioactive and therefore offers the exciting possibility that an *in vivo* evaluation of drug response may be completed in the population at large without radiation hazard to the individual. The relatively low precision of detection instruments and the requirement that relatively large amounts of isotope be given to overcome the high natural background of ^{13}C are important limitations to this use. However, I firmly believe that in many instances these limitations can be overcome. Clearly the successful application of this and other techniques described at the symposium will greatly increase the precision of medical diagnosis and they will add a most important new dimension to the concept of preventative medical care.

ACKNOWLEDGMENT

The author would like to gratefully acknowledge the contribution of Drs. Myron Pollycove and Matthew B. Fish who are located at the San Francisco General Hospital and University of California and with whom the studies of TCA cycle metabolism were initiated, and Drs. Roger Ryan, William Hofmann, Keith Brodie, and Vincent Zarcone of Stanford University who are presently collaborating on the development of these models and have contributed to the studies of L-dopa metabolism.

REFERENCES

1. Tolbert, B. M., Kirk, M., and Upham, F. Carbon¹⁴-Respiration Pattern Analyzer for Clinical Studies, *Rev. Sci. Instrum.* 30, 116-120, (1959).
2. Fish, M., DeGrazia, J. A., Pollycove, M., and Wallerstein, R. O. Abnormalities of Succinate Metabolism in Human Folate and Vitamin B₁₂ Deficiency, *Clin. Res.* 18, 184, (1970).
3. DeGrazia, J. A., Fish, M., Pollycove, M., and Fleury, K. Studies of the Metabolism of 3-¹⁴C Serine in Patients with Megaloblastic Anemia Due to Vitamin B₁₂ and Folic Acid Deficiency, (In preparation for publication).
4. DeGrazia, J. A., Fish, M., Pollycove, M., and Ponnampersuma, M. The Determination of Precursors to Urinary Methymalonic Acid in Man; Measurement of Urinary Excretion and Specific Activity, *Clin. Chim. Acta*, 23, 279-287, (1969).
5. DeGrazia, J. A., Fish, M., Pollycove, M., Wallerstein, R. O., and Hollander, L. The Role of Propionic Acid as a Precursor of Methylmalonic Acid in Normal and Vitamin B₁₂ -Deficient Man, *J. Lab. Clin. Med.*, 73, 917-923, (1969).
6. Hofmann, A. F., and Fromm, H. A New Breath Test for Bile Acid Deconjugation, *New Eng. J. Med.* 285, 686-687, (1971).
7. Fish, M. B., Pollycove, M., and Feichtmeir, T. V. Differentiation Between Vitamin B₁₂-Deficient and Folic Acid-Deficient Megaloblastic Anemias with C¹⁴-Histidine, *Blood* 21, 447-461, (1963).
8. Berlin, N. I., and Tolbert, B. M. Metabolism of Glycine-2-¹⁴C in Man: V. Further Consideration of Pulmonary Excretion of ¹⁴CO₂. *Proc. Soc. Exp. Biol. Med.* 88, 386-389, (1955).
9. Fish, M. B., Pollycove, M., and Wallerstein, R. O. *In Vivo* Oxidative Metabolism of Propionic Acid in Human Vitamin B₁₂ Deficiency. *J. Lab. Clin. Med.* 72, 767-777, (1968).
10. Gordon, E. S., and Goldberg, M. Studies of Energy Metabolism in Human Subjects Using Carbon¹⁴-Labeled Compounds. I. Effect of Sex, State of Nutrition, and Body Weight. *Metabolism* 13, 775-790, (1964).
11. Cozzetto, F. J. Radiocarbon Estimates of Intestinal Absorption. *Amer. J. Dis. Child* 107, 605-611 (1964).
12. Pollycove, M. Glucose Kinetics and Oxidation and the Effects of Insulin Tolbutamide, and Phenethylbiguanide DBI in Normal Human Subjects. Symposium of Respiration Pattern Analysis in Intermediary Metabolism Study, Applied Physics Corporation, 1964.

13. DeGrazia, J. A., Fish, M. B., Pollycove, M., Wallterstein, R. O., and Hollander, L. The Oxidation of the Beta Carbon of Serine in Human Folate and Vitamin B₁₂ Deficiency. *J. Nucl. Med.* 10, 329, (1969).
14. DeGrazia, J. A., MacLaury, M., Hofmann, W. W., Ryan, R., Fish, M. B., and Pollycove, M. *In Vivo* Studies of Citric Acid Cycle Metabolism in Man. *J. Nucl. Med.* 11, 314, (1970).
15. Van Praag, H. M. The Possible Significance of Cerebral Dopamine for Neurology and Psychiatry. *Psychiat. Neurol. Neurochir.* 70, 361-379, (1967).
16. Hornykiewicz, O. Dopamine (3-Hydroxytyramine) and Brain Function. *Pharmacological Reviews*. 18, 925-964, (1966).
17. Cotzias, G. C., Papavasiliou, P. S., and Gellene, R. Modification of Parkinsonism--Chronic Treatment with L-dopa. *New Eng. J. Med.* 280, 337, (1969).
18. Barbeau, A. L-Dopa Therapy in Parkinson's Disease: A Critical Review of Nine Years' Experience. *Canad. Med. Assn. J.* 101, 59, (1969).
19. Calne, D. B., Stern, G. M., and Laurence, D. R. L-Dopa in Post-encephalitic Parkinsonism. *Lancet* 1, 744-747, (1969).
20. Martin, W. E. Adverse Reactions During Treatment of Parkinson's Disease with Levodopa. *JAMA* 216, 1979-1983, (1971).
21. Lotti, V. J., and Porter, C. C. Potentiation and Inhibition of Some Central Actions of L-dopa by Decarboxylase Inhibitors. *J. Pharm. and Exptl. Therapeutics* 172, 406-415, (1970).
22. Goodwin, F. K., Brodie, K. H., Murphy, D. L., and Bunney, W. E., Jr. Administration of a Peripheral Decarboxylase Inhibitor with L-dopa to Depressed Patients. *Lancet* 1, 908-911, (1971).
23. Sourkes, T. L. L-dopa Decarboxylase: Substrates, Coenzymes, Inhibitors. *Pharmacological Reviews* 18, 53-60, (1966).
24. Chase, T. N. Cerebrospinal Fluid Monoamine Metabolites and Peripheral Decarboxylase Inhibitors in Parkinsonism. *Neurology*, 20, 36-40, (1970).
25. Bloise, M. S., and Taskovich, L. The Determination of L-dopa and L-dopa Metabolites using Diphenylpicrylhydrazine. In preparation for publication. Personal communication.
26. Bloise, M. S. Antioxidant Determinations by Use of a Stable Free Radical. *Nature* 181, 1199-1200, (1958).

27. Calne, D. B., Karoun, F., Ruthven, C. R., and Sandler, M. The Metabolism of Orally Administered L-dopa in Parkinsonism. *Brit. J. Pharmac.* 37, 57-68, (1969).
28. O'Gorman, L. F., Borud, O., Khan, I. A., Gjessing, L. R. The Metabolism of L-3,4-Dihydroxyphenylalanine in Man. *Clin. Chim. Acta.* 29, 111-119, (1970).
29. White, A., Handler, P., and Smith, E. L. Chapter 16, Biological Oxidations II. Citric Acid Cycle. The Mitochondrion, Electron Transport, Oxidative Phosphorylation. *Principles of Biochemistry*, McGraw Hill, 1968, p. 321-354.
30. Weinman, E. O., Strisower, E. H., and Chaikoff, I. L. Conversion of Fatty Acids to Carbohydrate: Application of Isotopes to this Problem and Role of the Krebs Cycle as a Synthetic Pathway. *Physiological Reviews* 37, 252-272, (1957).
31. Shreeve, W. W., Hennes, A. R., and Schwartz, R. Production of $^{14}\text{CO}_2$ from 1- and 2- ^{14}C -Acetate by Human Subjects in Various Metabolic States. *Metabolism* 8, 741-756, (1959).
32. Kaziro, Y., and Ochoa, S. Metabolism of Propionic Acid. *Advances in Enzymology* 26, 283, (1964).
33. Morrow, III, G., Barness, L. A., Cardinale, G. J., Abeles, R. H., and Flaks, J. G. Congenital Methylmalonic Acidemia: Enzymatic Evidence for Two Forms of the Disease. *Biochemistry* 63, 191-197, (1969).
34. DeHaan, E. J., and Tager, J. M. Evidence for a Permeability Barrier for α -Oxoglutarate in Rat Liver Mitochondria. *Biochim. Biophys. Acta* 153, 98-112, (1968).
35. Chappell, J. B., and Haaroff, K. N. The Penetration of the Mitochondrial Membrane by Anions and Cations. In E. C. Slater, Z. Kaniuga and J. Wojtczak. *Biochemistry of Mitochondria*. Academic Press and P.W.N., London and Warsaw, 1967, p. 75-91.
36. Hsia, D. Y., Berman, J. L., Justice, P., Nadler, H. L., and O'Flynn, M. E. Metabolic Disorders Associated with Mental Retardation. *Pediatric Clinics of North America*, 15, 889-904, (1968).
37. Fish, M. B., Pollycove, M., DeGrazia, J. A., Cohen, P., and Fleury, K. Effect of Route and Load of Administered Phenylalanine on Human *in vivo* Phenylalanine Catabolism. *J. Nuc. Med.* 9, 317, (1968).
38. Williams, R. H. The Pancreas. *Textbook of Endocrinology*, R. H. Williams, ed. W. B. Saunders, Philadelphia and London, 1962, p. 559-613.

TABLE I

BREATH PARAMETERS OBTAINED AFTER ADMINISTRATION OF 1-¹⁴C L-DOPA TO CONTROL SUBJECTS AND PATIENTS RECEIVING THERAPY WITH MK486 AND/OR L-DOPA

	T _{max} (min)	Cumulative ¹⁴ C 1/2 hr	Exhaled as % of Dose 1 hr	2 hr
Normal (no drugs) (3)*	<u>5.7</u> (4-7)	<u>16.1</u> (12.4-19.5)	<u>25.5</u> (20.6-30.7)	<u>37.6</u> (31.0-44.6)
Parkinson (no Dopa) (1)	<u>6</u>	<u>18.3</u>	<u>30.4</u>	<u>42.0</u>
Normal (5HTP, 2.5 g q.d.) (3)	<u>5.3</u> (5-6)	<u>14.5</u> (13.7-15.3)	<u>24.1</u> (23.1-24.5)	<u>37.1</u> (36.1-37.9)
Parkinson (Oral L-Dopa, 4-6 g q.d.) (6)	<u>6.5</u> (6-7)	<u>15.3</u> (12.0-18.1)	<u>26.4</u> (21.1-29.6)	<u>39.1</u> (35.0-44.2)
Normal:MK486				
6 25 mg 4X day (1)	<u>10</u>	<u>6.4</u>	<u>12.0</u>	<u>20.0</u>
12.5 mg 4X day (1)	<u>10</u>	<u>5.1</u>	<u>9.5</u>	<u>16.0</u>
25 mg 4X day (2)	<u>10.5</u> (9-12)	<u>5.3</u> (4.6-5.9)	<u>9.6</u> (8.7-10.5)	<u>15.8</u> (14.7-16.8)
50 mg 4X day (2)	<u>12</u> (10-14)	<u>4.6</u> (3.9-5.3)	<u>8.6</u> (7.6-9.5)	<u>13.9</u> (12.9-14.9)
Normal:MK486 + Dopa, 2 g q.d. (1)				
	<u>14</u>	<u>4.3</u>	<u>8.3</u>	<u>14.6</u>
Parkinson:MK486 + Dopa, 2 g q.d. (2)				
	<u>16</u> (15-18)	<u>4.2</u> (3.5-4.8)	<u>7.6</u> (7.0-8.3)	<u>13.2</u> (12.5-14.0)

*Numbers in parentheses represent number of studies.

TABLE II

FIVE NORMAL VOLUNTEERS: DATA AFTER I.V.

1,4-¹⁴C-SUCCINATE AND 2,3-¹⁴C SUCCINATE

1,4- ¹⁴ C Succinate		Cumulative % ¹⁴ C		
Patient	T _{max}	1/2 hr	1 hr	2 hr
R. G.	5	28	42	60
A. J.	5	22	33	51
M. L.	6	20	32	48
E. S.	5	22	36	59
I. W.	6	25	39	59
Range	5-6	20-28	32-42	48-60
2,3- ¹⁴ C Succinate				
R. G.	20	6.9	14	25
A. J.	20	5.0	10	20
M. L.	24	5.8	12	23
E. S.	21	4.4	9	19
I. W.	36	4.7	11	21
Range	20-36	4.4-6.9	9.0-14	20-25

TABLE III

BREATH PARAMETERS OBTAINED AFTER INTRAVENOUS ADMINISTRATION
OF RADIOCARBON LABELED TCA CYCLE INTERMEDIATES
TO CONTROL AND L-DOPA TREATED SUBJECTS

Normal Subjects	T _{max}	1/2 hr	Cumulative % ¹⁴ C ₂ 1 hr	2 hr
5- ¹⁴ C-α-keto- glutarate (3)*	<u>15</u> (12-17)	<u>14</u> (12-14)	<u>25</u> (23-26)	<u>42</u> (41-44)
1,4- ¹⁴ C-succinate (5)	<u>5.6</u> (5-6)	<u>23</u> (20-28)	<u>37</u> (32-42)	<u>54</u> (47-60)
1- ¹⁴ C-acetate (1)	<u>11</u>	<u>17</u>	<u>32</u>	<u>56</u>
2,3- ¹⁴ C-succinate (5)	<u>24</u> (20-36)	<u>5.1</u> (4.1-6.9)	<u>11</u> (9.3-1.4)	<u>21</u> (19-25)
2- ¹⁴ C-acetate (1)	<u>60</u>	<u>2.3</u>	<u>6.2</u>	<u>15</u>
Parkinson on L-dopa +				
5- ¹⁴ C-α-keto- glutarate (3)	<u>18</u> (15-21)	<u>9.2</u> (7.1-12.4)	<u>18</u> (15-24)	<u>32</u> (27-40)
1,4- ¹⁴ C-succinate (5)	<u>6.6</u> (6-7)	<u>18</u> (11-26)	<u>28</u> (20-40)	<u>42</u> (31-57)
1- ¹⁴ C-acetate (3)	<u>13</u> (10-17)	<u>7.7</u> (6.4-10)	<u>15</u> (12-21)	<u>28</u> (23-26)
2,3- ¹⁴ C-succinate (4)	<u>28</u> (22-36)	<u>3.4</u> (2.4-4.2)	<u>7.8</u> (5.6-9.1)	<u>16</u> (12-17)
2- ¹⁴ C-acetate (4)	<u>58</u> (42-84)	<u>2.5</u> (2. 2.5)	<u>6.3</u> (5.4-7.2)	<u>14</u> (13-17)

*Numbers in parentheses refer to numbers of cases.

TABLE IV

RADIORESPIROMETRY PARAMETERS OBTAINED AFTER ADMINISTRATION OF 1-¹⁴C-L-DOPA, 1,4-¹⁴C SUCCINATE, 2,3-¹⁴C SUCCINATE AND 1-¹⁴C L-TYROSINE TO PARKINSON PATIENTS PRIOR TO AND DURING THERAPY WITH MK486*

	T _{max} (mins)	% Dose Recovery in		
		1/2 hr	1 hr	2 hr
L-Dopa				
Before MK486 (2)	<u>5.5</u> (5-6)	<u>17.1</u> (14.6-19.6)	<u>27.2</u> (23.7-30.7)	<u>39.2</u> (33.4-45.1)
After MK486 (2)	<u>11.5</u> (11-12)	<u>4.0</u> (3.55-4.45)	<u>7.19</u> (6.36-8.03)	<u>11.6</u> (10.6-12.7)
1,4- ¹⁴ C-Succinate				
Before MK486 (2)	<u>6.5</u> (6-7)	<u>22.5</u> (19.8-24.9)	<u>35.0</u> (31.3-39.3)	<u>49.1</u> (42.4-56.5)
After MK486 (2)	<u>6.0</u> (5-7)	<u>22.3</u> (19.8-24.9)	<u>32.9</u> (27.0-38.6)	<u>49.1</u> (40.2-58.6)
2,3- ¹⁴ C-Succinate				
Before MK486 (1)	<u>22.0</u>	<u>4.15</u>	<u>9.05</u>	<u>17.3</u>
After MK486 (1)	<u>27.0</u>	<u>3.43</u>	<u>8.54</u>	<u>17.1</u>
1- ¹⁴ C-Tyrosine				
Before MK486 (1)	<u>8.0</u>	<u>2.44</u>	<u>4.46</u>	<u>7.40</u>
After MK486 (1)	<u>9.0</u>	<u>2.04</u>	<u>3.69</u>	<u>5.81</u>

*All subjects received 2 g of L-dopa daily in control and treatment periods.

Figure 1. Stanford metabolic gas analyser.

Figure 2. Graphics display and computer terminal. Data is collected by a time shared disk. When the patient testing is completed, the results are computed and may be immediately displayed in the format shown.

Fig. 3, P. 90

Figure 3. The $^{14}\text{CO}_2$ expiration pattern obtained when $1\text{-}^{14}\text{C-L-dopa}$ was given to a control subject. This figure graphically illustrates the parameters calculated and the fitting function used to compute the radiorespirometry curve.

Figure 4. Breath specific activity after intravenous administration of five radiocarbon-labeled amino acids to control subject.

Figure 5. Breath specific activity after intravenous administration of 1,4-¹⁴C succinate and 2,3-¹⁴C succinate to a control subject.

Figure 6. Metabolism of L-dopa to dopamine. MK486 will reduce CO₂ production in this reaction by lowering dopa decarboxylase activity in tissues other than brain.

Figure 7. Serial studies of a control subject showing the progressive reduction in breath specific activity that results as MK486 dosage is increased.

Figure 8. Serial studies of a control subject showing the failure of breath specific activity to fall further as the dose of MK486 is increased from 50 mg. to 200 mg daily.

Figure 9. The cumulative recovery of radiocarbon in breath (2 hours) and urine (26 hours) after intravenous administration of $1\text{-}^{14}\text{C}$ -L-dopa to control subjects receiving varied amounts of L-dopa or L-dopa and MK486.

Figure 10. Total urinary L-dopa excretion by control subjects receiving varied doses of MK486 or L-dopa plus MK486 (26 hour collection).

Figure 11. The fractional excretion of radiocarbon in urine following the administration of 1-¹⁴C-L-dopa to control subjects receiving varied doses of MK486 (26 hour collection).

Figure 12. Urinary specific activity following the administration of $1\text{-}^{14}\text{C}$ -L-dopa to two control subjects receiving varied amounts of L-dopa or L-dopa and MK486 (26 hour collection).

Figure 13. The metabolism of L-dopa emphasizing the possible distribution of the carboxyl carbon to metabolites excreted in urine and the alternate pathway for phenylalanine and tyrosine oxidation via the TCA cycle.

Figure 14. A comparison of the $^{14}\text{CO}_2$ respirometry curves obtained after intravenous administration of 1,4- ^{14}C succinate, 2,3- ^{14}C succinate, or 1- ^{14}C acetate to a normal control subject.

Figure 15. The turnover of radiocarbon within the TCA cycle from 1,4- ^{14}C succinate, 2,3- ^{14}C succinate, 1- ^{14}C acetate and 2- ^{14}C acetate. Note that collectively these compounds trace the turnover of all the carbon atoms of citrate. However, only two of these carbon atoms are removed as CO_2 during each passage of citrate through the cycle. The remaining carbon atoms re-enter the cycle as succinyl CoA. They are then removed on repeated recycling.

Figure 16. The distribution of radiocarbon from propionate to succinyl CoA. Note that carboxyl-labeled succinate enters the TCA cycle effectively as 1,4-¹⁴C succinate due to "randomization."

Figure 17. Radiorespirometry data from a single patient and the breath parameters calculated from comparative studies of control subjects receiving 1,4-¹⁴C succinate, 2,3-¹⁴C succinate, 1-¹⁴C propionate and 2-¹⁴C propionate. All subjects were maintained on a constant diet and hospitalized in the Clinical Research Center for the duration of these studies.

Fig. 18, P. 98

Figure 18. Serial studies of a normal control subject demonstrating the effect of phenylalanine loading upon the $^{14}\text{CO}_2$ expiration pattern.

INSTRUMENTATION - MASS SPECTROSCOPY

Chairman: K. Biemann

THE MEASUREMENT OF ISOTOPE RATIOS IN ORGANIC MOLECULES BY MASS SPECTROSCOPY

Peter D. Klein

A ^{13}C EXPERIMENT USING MASS SPECTROMETER BREATH ANALYSIS

B. B. McInteer

AN *IN VIVO* STUDY OF THE METABOLISM OF HUMAN PLASMA GLYCOSPHINGOLIPIDS USING 6,6 DIDEUTERIOGLUCOSE AS A TRACER

Dennis E. Vance and Charles C. Sweeley

THE MEASUREMENT OF ISOTOPE RATIOS IN ORGANIC MOLECULES
BY MASS SPECTROSCOPY*

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Measurements of the amounts of stable isotopic labeling in organic compounds can be carried out by many alternatives to mass spectroscopy, but these have in common the limitations of sample size, limited speed or limited sensitivity. For the purposes of this seminar, the minimum capabilities for isotope ratio measurements to be considered are a 10 μ g sample, with a dilution of the original label to 1 part in 10^3 , measured within a 10 minute period. To date, mass spectrometry is the only technique which attains and significantly exceeds these requirements. In this discussion, I would like to describe the types of instruments used for these measurements, examine some of the applications of stable tracers and the dilution or sensitivity required, and identify the bottlenecks in any development of sound, inexpensive, commercial instruments for clinical pharmacology.

All mass spectrometers, whether electromagnetic or quadrupole, physically segregate ionized molecules or ion fragments according to mass before they are detected and recorded. If the entire series of ion groupings is recorded, the result is a *mass spectrum*; if two selected ions are compared, the result is an *isotope ratio*. The simplest system for measuring isotope ratios thus has two fixed collectors, each positioned to intercept the appropriate mass, e.g., 44 and 45 for $^{12}\text{CO}_2$ and $^{13}\text{CO}_2$ respectively. Such *dual collector* instruments provide continuous readings of each mass independently but suffer from the limitations of predetermined and fixed focus and from the limited sensitivity of the Faraday cup detectors as compared with electron multiplier detectors. Clearly, they provide no structural information whatsoever. In the second category of isotope ratio systems, the focus is switched between two, three or more ions by stepping the accelerating voltage down in increments such that the next higher ion of interest strikes the detector. Such an *accelerating voltage alternator* (1-3) (AVA) or *multiple ion detector* (MID) (4)

*This work was supported by the U. S. Atomic Energy Commission.

uses the greater sensitivity of the electron multiplier to detect small abundances of ions and can be used to compare masses that differ by as much as 50%. It may be used to provide a continuous *ion specific record* (4,5) or for digital values of isotope ratios (3) and has the advantage of complete flexibility in the selection of ions to be monitored. It provides specific but limited structural information. The third system requires the use of a dedicated computer to acquire the information generated by a *repetitive scanning* mode of operation (6). All ion intensities in the interval of masses being scanned are recorded and stored for each scan, and after the required number of scans have been completed, the computer is used to retrieve the selected ion(s) from each scan. This retrospective search capability permits one to examine a large number of ion records from a single sample and thus provides an enormous amount of structural information. Some examples of the use of an AVA system and a repetitive scanning system are shown in Figures 1-4.

There are four categories of application of stable isotopic tracers, each successively more demanding in the sensitivity of measurement required. Because each stable isotope occurs with characteristic natural abundance that constitutes the background against which "atoms percent excess" is measured, it is convenient to speak of the *dilution* to which the original labeled molecule is subjected and to characterize the applications by the magnitude of the dilution at which isotope concentration need be measured.

The first category, which will be described tomorrow in greater detail by Dr. Thomas Gaffney, is the use of stable isotopic labeling to permit recognition of drug metabolites in urine or body fluids (5). This technique uses the multiple ion detector to screen a gas chromatographic effluent for compounds having a characteristic ion pair in their spectrum. One such ion pair might be produced by an isotopically substituted oxygen atom in which half of the atoms are ^{16}O and half are ^{18}O . This substitution results in a doublet separated by two mass units in which the ions are of equal intensity, and so long as the portion of the molecule in which the labeled atom is present remains intact, the appearance of a doublet at these two masses in the ion specific record will mark the emergence of a product arising from the original drug molecule. Such applications result in relatively little dilution of the labeled molecule (10^0 - 10^{-1}) since metabolic processes usually do not contribute to the *de novo* production of metabolite, and structural information about the metabolite is usually far more valuable than measurement of its concentration. In this regard and at this dilution, the stable isotopic tracer acts as a qualitative, not quantitative, identification tool.

The second category is that of kinetic studies such as those described by Dr. Alan Hofmann this morning and by Dr. Roger Lester tomorrow morning, in which pool sizes and replacement rates are determined. These types of studies will usually tolerate the addition of the labeled metabolite in quantities up to 10% of the pool size without disturbing the kinetic measurements. During the study, the tracer will be present in dilutions of 10^{-1} to 10^{-3} ; only the isotope

present in the original, known, structure needs be measured and thus structural information is not required.

The third category, metabolite quantitation by inverse isotope dilution, is of increasing importance in the measurement of tissue constituents at the nanogram level, such as the prostaglandins, and will be discussed in greater detail by Dr. Throck Watson and Dr. Charles Sweeley. This procedure calls for the addition of an isotopically labeled carrier to a tissue extract and the subsequent measurement of the ratio of labeled to unlabeled compound, usually at dilutions of 10^{-2} to 10^{-4} (7). Such techniques have serious competition from derivatization with electron capture reagents, but possess the advantages of high specificity and relative freedom from the necessity for quantitative recovery or other internal standards. The limitations arise from the cross contribution of labeled to unlabeled ion background intensities which may equal or exceed the ion intensity of the unlabeled compound present.

The final category is that of metabolic studies such as those described by Dr. William Nyhan in which there is a dismutation of the labeled molecule and dispersion into many metabolites at high dilution, all of which may eventually find their way to respiratory CO_2 , water, or urea. Detection of the label in these end products may require the measurement of dilutions as low as 10^{-4} to 10^{-6} , but even more stringent is the requirement that such studies eventually need the ability to detect isotope in *any* compound of known or unknown structure. This remains the greatest challenge to instrument development.

The measurement of isotopic dilution is a process not unlike radiocarbon dating, in that there is an upper limit to the abundance or activity present in the sample and there is a background abundance/activity which is present in all samples. The sensitivity is thus related to the magnitude of the change in the natural abundance that can be detected. For example, ^{13}C occurs with an abundance of 1.108% that of ^{12}C and if we are able to begin with a compound that has its carbon isotope content enriched to 90%, and are able to detect a change in the natural abundance from 1.108 to 1.208 (10%), then we are able to measure a dilution of the original label to slightly more than 1×10^{-3} . It is readily seen that the requirements grow more stringent the greater the dilution that we seek to measure; for example, dilutions to 1×10^{-5} would require the ability to differentiate between a natural abundance of 1.108 and 1.109% ^{13}C .

How is abundance measured and what determines the limits to which dilution can be measured? In essence, the ion intensities of normal and heavy isotopic ions must be measured with the needed accuracy within the time that the sample is present in the mass spectrometer. This is done by sampling the ion current and converting the analog signal to a digital value: the ultimate limitation lies in the speed and accuracy with which this conversion is carried out. For example, a 10 bit analog to digital converter has a full scale digital output of 1024 counts and the uncertainty of the ion intensity is proportional to the square root of the number of counts accumulated. Table I lists some examples of the numbers of counts that must be accumulated to achieve a given level of uncertainty and the corresponding dilutions to which $^{13}\text{CO}_2$ could be

measured under such circumstances. The exponential increase in the number of counts required to measure higher and higher dilutions is evident and underscores the fact that *counting rates* are the ultimate limitation in stable isotope abundance determinations.

Table I

Relationship of Dilution, Counting Error and Total Counts
Required for the Determination of $^{13}\text{CO}_2/^{12}\text{CO}_2$ Isotope Ratios

Dilution of $^{13}\text{CO}_2$ to be Measured	Permissible Error in Ratio	Total Counts Required to Establish Ratio
1×10^{-3}	10%	100
1×10^{-4}	1%	10^4
1×10^{-5}	0.1%	10^6
1×10^{-6}	0.01%	10^8
1×10^{-7}	0.001%	10^{10}

How fast might these counting rates be? There are commercially available 15 bit analog to digital converters that can measure and convert an analog signal within 50 μsec ; ideally this would mean a counting rate of $32,000 \times 20,000$ or 6.4×10^8 per second. Not all of this is usable, however, for several reasons. First, if we allow headroom for the gas chromatographic peak in which the isotopic abundance is being measured, and take into account that most amplifiers are attenuated in steps of 2x, a usable range of 10,000 of the 32,000 full scale seems likely. Second, both light and heavy ions must be measured to obtain the abundance, so we must either use two A/D converters or devote half of the counting time to the reference ion, which does not contribute to the accuracy with which the isotopic ion is measured. Third, a small increment, perhaps 10%, is lost due to switching and settling time between light and heavy ions. These three factors combine to reduce the usable counting rate to approximately $8 \times 10^7/\text{sec}$. If the sweep mode is used instead of static switching from peak maximum to maximum (3) the counting rate will be used with only 25% efficiency, since the peak occupies only a quarter of the total area in the sampling interval. There is a further reduction in counting rate if there is no attenuation of the light ion intensity, for if both ions have to be sampled at the same amplification, only a small portion of the counting rate is used to measure the less abundant heavy ion. These relationships are summarized in Table II and indicate that counts of 10^8 may be accumulated within a time interval of 0.5-4.0 seconds under the best conditions. There is a substantial increase in time required if other structural information is recorded at the same time.

Table II

Relative Counting Speeds of Mass Spectrometers Intended for
Isotope Ratio Measurements

Type	Time, 10^8 Cts	Comments
Dual Collector	0.5 seconds	Individual A/D units
	1.0 seconds	Shared A/D unit
Accelerating Voltage Alternation	1.1 seconds	Step Switching, allowing 10% settling time
	4.0 seconds	Sweep mode vs step mode
Multiple Scanning	10^4 scans	For full scale peak
	10^6 scans	For 1% intensity peak

The structure of the isotopically labeled molecule also plays an important role in the accuracy with which isotopic composition can be measured. For example, the singly labeled ^{13}C compound gains in background at the rate of 1.1% for every carbon atom in the structure; thus a C_{26} bile acid has a background that is 30% of the parent ion for the intact molecule (that is, the $M+1$ ion, which represents the ^{13}C -labeled molecule is already one third as large as the unlabeled M ion, *without the addition of any labeled molecules*). This requires a counting time which is $(30)^2$ or 900 times longer than for the measurement of a comparable accuracy in $^{13}\text{CO}_2$ vs $^{12}\text{CO}_2$. This can be overcome in the intact molecule by multiple labeling which reduces the background of the labeled peak (which now becomes $M+2$, $M+3$ or $M+4$) as the power of the number of labeling atoms present. This involves more extensive synthesis than single labeling however. A second factor is that a large molecule fragments into many ions in the usual electron impact ion source. Chemical ionization may reduce this tendency but may also lose total ionization at the same time. The result is that the isotope is dispersed among many fragments and the intensity of any one ion available for quantitation is proportionately less. Finally, the fragmentation process may lead to the formation of pseudo-labeled ions, ions which have the same molecular weight as the isotopically labeled ion, but that arise from higher fragments by secondary fragmentation patterns and that are not the isotopic ion.

The profound effects of structure on the accuracy of isotopic ratio measurements are illustrated in Figures 5-9 which contrast the spectrum of 2,2,4,4, ^2H -chenodeoxycholic acid and the dilution limits to

which the labeled bile acid can be detected, with the spectrum of 1,3,¹⁵N-pentobarbital and its dilution curve and finally the dilution curve for ¹³CO₂, all measured on the same instrument with the same counting system. The spectrum for the tetradeuterio bile acid is complex, and though there are four labeled atoms per molecule, the limit of dilution is near 0.2%. The barbiturate spectrum is characterized by much greater simplicity and the *doubly* labeled ¹⁵N compound can be detected to concentrations of 0.02%, or a factor of 10 greater than the bile acid. Finally, ¹³CO₂ has been measured to concentrations of approximately 0.005% above normal and in recent studies such as Dr. Nyhan's, this has been reduced to 0.002%.

The implications of these figures seem quite clear: Sensitivity of isotope ratio measurements is gained at the expense of structural information and *vice versa*. While acquiring one category of information does not preclude obtaining the other, in the last analysis, they are separate objectives and should neither be confused nor combined if maximum efficiency for either is desired.

It also seems clear that mass spectrometers designed to obtain both mass spectra and isotope ratio measurements are likely to be more expensive and less satisfactory than instruments which do one or the other. Taken at face value, Figures 6, 8 and 9 suggest that ¹³C should be determined as CO₂, ¹⁵N as N₂ and ²H as H₂. Such forms of the isotopes require only a limited mass separation capability that can be easily satisfied by an inexpensive leak-detector type instrument. If the separation processes of a gas chromatograph can be successfully coupled with the mass spectrometer through an intervening combustion train such as the Dumas nitrogen analyser, (8) the intrinsic isotope content of any compound should be measurable with good to high sensitivity. Such an instrument would go a long way toward replacing ³H, ¹⁴C and scintillation counting in present tracer methodology. It would be competitive in cost with liquid scintillation counting, which the present generation of GC-MS-AVA are not. It is for this reason that I believe that the development of reliable inexpensive instrumentation, based on this or any other concept, is the number one priority in the application of stable isotopes to clinical pharmacology in particular and to medicine as a whole.

References

1. C. C. Sweeley, W. H. Elliott, I. Fries and R. Ryhage. *Anal. Chem.* 38, 1549 (1966).
2. C. G. Hammar, B. Holmstedt and R. Ryhage. *Anal. Biochem.* 35, 532 (1968).
3. P. D. Klein, J. R. Haumann and W. J. Eisler. *Anal. Chem.* 44, 490 (1972).
4. C. G. Hammar and R. Hassling. *Anal. Chem.* 43, 298 (1971).
5. T. E. Gaffney, C. G. Hammar, B. Holmstedt and R. E. McMahon. *Anal. Chem.* 43, 307 (1971).
6. R. Hites and K. Biemann. *Anal. Chem.* 42, 855 (1971).
7. M. Minton and P. D. Klein. This conference, p. 265.
8. P. D. Klein, P. A. Szczepanik and A. F. Hofmann. Proceedings of the National Carbon-13 Symposium, Los Alamos, N. M. June 9-11 (1971). In Press.

Figure 1. Continuous recording at m/e 217 and 220 during elution of TMSi glucose and glucose-d₇ mixture from 3% SE-30 column (1).

Figure 2. Digital representation of gas chromatographic peak obtained from m/e 374 (tetra-deuterio chenodeoxycholic acid) and from m/e 370 (protio form). Dotted line: isotope ratio from integrals of individual ion peaks (3).

Figure 3. Upper panel: Mass spectrum of trifluoracetylated nortriptyline. Lower panel: Ion specific recordings of 50 ng of trifluoracetylated nortriptyline. (4).

Figure 4. Total ionization plot (*—) and mass chromatograms of $m/e=74$ (.....) and 98 (----) of methyl esters of a non-steam distillable acidic extract of Green River oil shale (6).

Figure 5. Mass spectrum of methyl chenodeoxycholic acid (3).

Figure 6. Dilution curve for mixtures of d_0 and d_4 -labeled chenodeoxycholic acid (3).

Figure 7. Mass spectrum of N,N',dimethylpentobarbital (7).

Figure 8. Dilution curve for 1,3-¹⁵N dimethyl pentobarbital (7).

Figure 9. Dilution curve for ¹³CO₂ (8).

DISCUSSION

Chairman Biemann: We will start out with questions which are specifically related to Dr. Klein's paper and which might be answered best by him.

Dr. Weiger: What is the cost of improving a mass spectrometer with the AVA?

Dr. Klein: The system which we have described, has been designed for the Perkin-Elmer mass spectrometer, but also has been adapted for the LKB instrument. I think Dr. Wall can attest to this. He is buying one from our Electronics Division, with a lot of gorgeous things on it, and it is running about \$15,000.

Dr. Wall: That's right.

Dr. Sweeley: I would like to say that I think the potential here is very great. This piece of gear which Peter has built is very fine for doing about as accurate a measurement of isotope ratio as I have seen, but I think as you go into the utilization of a computer for control as well as acquisition, with a feedback loop and sending signals to control voltage and ramping, it will be possible to duplicate your experiments for about \$500 worth of A/D converter and feedback loop.

Chairman Biemann: If I may make a comment, I think there are two areas. It is not only a matter of price: a simple black box for a little money and a very elaborate black box for a lot of money, you also have two completely different problems.

The entire carbon-13 discussion started out with the desire to replace carbon-14 as a general tracer, and for this you need instruments to measure carbon-13 in a generally applicable technique for a little money, just like counting carbon-14. What we are talking about here with this AVA-computer controlled mass spectrometer, which would be a reasonably elaborate instrument, doesn't fall into that category. If you want to pin down a specific molecule you might as well take the big instrument, do it once, and get it over with.

Dr. Wall: In terms of cost, people working with radioisotopes think nothing of buying scintillation counters, which I would say nowadays probably run around \$15,000. We find these in biochemical and pharmacology groups, not by the ones but sometimes by the threes.

Chairman Biemann: That is comparable to the low cost mass spectrometer.

Dr. Wall: That is what I meant. There are two levels; between \$5,000 to \$20,000 is one level, and between \$50,000 and \$250,000 is another level.

Dr. Sweeley: That might be a little unfair too, because the price of computers is coming down drastically, and so is the price of mass spectrometers that are capable of doing AVA work. You can couple some of the instruments in the quadrupole class, with a PDP-8 for a cost much less than \$60,000 to \$250,000 and I can foresee the ability to do these analyses much more inexpensively in the next couple of years.

Dr. Levine: I think I heard you say that you could detect the bile salt that was labeled with four deuteriums at a concentration of one part per 500. This is the concentration in the final solution that was prepared to put into the machine. What sort of blood level or level in some sort of tissue does that actually represent? Would you have to have one part per 500 in blood, or 5,000 or 50,000?

Dr. Klein: The concentration is the same, regardless of where the sample comes from. It then hinges on how small a sample you need to make the measurement, which I think is what you are asking. Perhaps 5 μg would be a good number. You might push it to 1 μg . You might want to do it in very high dilution to 10 μg . It is not the concentration in the sample; that remains limited, let's say one in 500, but how much sample it takes to measure that dilution.

Dr. Levine: So in your final assay the material that ends up going into the mass spectrometer would have to attain 5 μg of--

Dr. Klein: --of which 1/500th is the label.

Dr. Wall: Five μg of total sample - what does that mean in terms of actual ions? Won't there be heavy losses in the molecular separator?

Dr. Klein: That's right.

Chairman Biemann: But the ratio doesn't change. He said you put in 5 μg and you see one in 500 ratio. Of course, one of the problems with going to lower and lower sample sizes is that the impurities and unavoidable instrument background become more and more prominent.

Dr. Wall: This is a premature question, I think, but will we have some time during the discussion to compare the relative merits of the AVA systems versus continuous scan? Are you going to do that, Dr. Biemann? I hope there will be some discussion on that.

Chairman Biemann: On the list of instruments Dr. Klein showed, the continuous scanning really was misplaced. If you want to look at two masses you would not scan from mass 20 to mass 650 repeatedly. It was actually a method to do other things. It certainly doesn't, by definition, reach the same sensitivity as the other techniques.

Dr. E. C. Horning: I wonder if you or Dr. Klein would comment on another point in greater detail. At the present time most of our work is done with a repetitive scan, looking for metabolites. This works very well for several reasons that have been mentioned. As far as the measurements of isotope ratios are concerned, we would very much like to use Dr. Klein's circuits because they seem to be the best so far.

I would wonder whether there is in principle any difference in ultimate sensitivity between either the hardware approach or the repetitive scan approach using software and scanning, say, over two or three masses, or at least a very short mass range. It seems to me the ultimate sensitivity ought to be the same if one does it by the software approach or by the hardware approach.

Chairman Biemann: That would be--jumping from 369 to 375, then you can scan instead of jumping.

Dr. Klein: Right; but now I think you are coming down to the AVA in the sense that you are throwing away all the other structural information which you have acquired. That is quite feasible; but it also illustrates what I am saying--that the tradeoff is between structural information and isotope ratio information; and the more you get of one, the more you have to relinquish of the other.

Chairman Biemann: You can get a better isotope ratio. If you don't know what you are looking at, you have to be content with less accuracy but with finding out what you are looking at. I think in general one will be followed by the other. First you find out what you have to look for, and then you do a measurement on that.

Dr. Wall: Right. You are not going to be using AVA techniques on an unknown--or at least I haven't heard about it.

Dr. Sweeley: I have several slides that illustrate the AVA method for quantitative analysis by combined gas chromatography-mass spectrometry. The technique is in a way the reverse of what Dr. Klein has been talking about, in that we are trying to measure very small amounts of unlabeled species. To prevent adsorption in the gas chromatography column a stable isotope-labeled form of the compound or its derivative is added as a carrier substance. Since it is also an internal standard it is possible to carry out quantitative analyses by measuring the isotopic ratio, and this method is potentially capable of very high sensitivity.

One of the applications of this technique, which Dr. Bengt Samuelsson and I worked on last year in Stockholm, involves analysis of glucose. The formula for the trimethylsilyl methoxime derivative of D-glucose is shown in Figure 1. This derivative is excellent for gas chromatography of monosaccharides and also provides a means of

Figure 1. Structure of 2,3,4,5,6-penta-O-trimethylsilylglucose methoxime and fragment ions observed in the mass spectrum of this compound.

Figure 2. Partial mass spectra of the protium and d_3 species of trimethylsilylglucose methoxime, labeled at the methoxime O-methyl group.

Figure 3. Mass spectrometric determination of the d_3 and protium forms of trimethylsilylglucose methoxime by the AVA technique.

Figure 4. Structure of the trimethylsilylmethyl ester of a d_4 form of $\text{PGF}_{2\alpha}$ used as carrier and internal standard for the quantitative analysis of prostaglandin by AVA technique (described in more detail in Samuelsson, Hamburg, and Sweeley, *Anal. Biochem.* 38, 301 [1970]).

Figure 5. Mass spectrometric determination of PGF_{2α} at the nanogram level by the AVA technique with a d₄ form as carrier and with computer acquisition and reduction of data (C. C. Sweeley, J. F. Holland, R. Thrush, R. Teets, and M. Bieber, in preparation).

preparing a labeled species as carrier, involving use of D_3 -methoxylamine as a reagent. Figure 2 shows the partial mass spectra of the protium and trideuterated forms, and indicates that analyses of mixtures could be made with a scanning or AVA technique to monitor the ion intensities at m/e 160 and 163. Contributions of neighboring ions to blanks are very low in the spectra and it is possible to obtain reasonably accurate isotope ratios with very small amounts of the protium form.

The results that Samuelsson and I obtained with mixtures of the d_3 and unlabeled forms of the glucose derivative are shown graphically in Figure 3. When 45 ng of the trideuterated form were injected into the LKB 9000 instrument, as little as 3 pg of the protium form per ng of labeled species could be measured. The limit of detectability for injections of this size was therefore 135 pg (3×45). The dotted line in Figure 3 represents data from injections of only 7 ng of the mixture. If the limiting proportion that can be measured accurately is the same--that is, 3 pg of protium form per ng--the limit would be about 20 pg of the protium form of glucose that could be determined with this method.

The structure of a prostaglandin recently synthesized by the Upjohn Company is shown in Figure 4. In this case the labeled form of $PGF_2\alpha$ is utilized rather than a derivative which contains the label in the extra functional group(s) of the derivative. This has the advantage that the labeled form can be added directly to plasma or urine and serves as a carrier and internal standard for the entire isolation procedure, not just for the GLC-mass spectrometry step. The relative intensities at m/e 427 for the tetradeuterated form and at m/e 423 for the corresponding protium form were monitored in analyses of $PGF_2\alpha$, ions representing (M-71-90).

A graphical representation of the results obtained with standard mixtures of labeled and unlabeled prostaglandin species is shown in Figure 5. A dedicated small digital computer was used for automatic control of the accelerating voltage to maintain accurate focus of the ions and also for data acquisition and reduction (details of the computer version of our AVA system are presently in preparation for publication with my colleagues, J. Holland, R. Thrush, R. Teets, and M. Bieber). It was possible to determine as little as 1 ng of $PGF_2\alpha$ (protium form) per μg of the d_4 carrier added, which represents analyses of 1000:1 mixtures. If 100 ng of the mixture were injected this would represent a capability for quantitative analyses in the range from 100 to 10,000 pg of $PGF_2\alpha$.

Dr. DeGrazia: I wonder if there is any other way to do these analyses. Is this the limit in the way of instrumentation? I am thinking of molecular excitation. Is there any possibility that with this we might be able to increase the sensitivity of this method?

Dr. Sweeley: The only thing I know of that approaches this in sensitivity and perhaps specificity is the radioimmunoassay.

Dr. DeGrazia: I am actually thinking of measuring carbon-13. What other possibilities are there? Are there none?

Dr. Klein: In terms of sample size? You can measure smaller quantities of carbon-13 under certain specialized conditions, but not under the conditions of these metabolic experiments. I don't know of any other practical way to do it. One of the things that confers a considerable advantage on the mass spectrometer is that you can put it behind a gas chromatograph. Anything you put into a gas chromatograph will go into the mass spectrometer. Sample handling is much, much simpler. As a matter of fact, you don't even have to have it pure. The whole tendency that has brought back both mass spectrometry as a practical thing, and the realistic application of stable isotopes has been this coincidence in the development of instruments.

Dr. DeGrazia: What is the problem there, specifically?

Dr. Klein: If you have to handle a nanogram of a compound and it is in a lot of other crud, you have a problem. You have to see that amount amidst all the other junk.

Dr. DeGrazia: The reason I am bringing this up, obviously, is this: what other possibilities are there if we are going to design a new instrument? What is the frequency of sampling going to be?

Chairman Biemann: That touches on what I said before, that the question is, what type of instrument is suitable for analysing 1,000 samples a day, or let's say 50 samples a day, every day, in any hospital? Then GC/MS is not so easy as it may sound. If one is working with one patient who has a very peculiar disease and one wants to figure that out, and can spend a few weeks or months solving that problem, and has the equipment available, one can do it. But we always have to keep these two objectives separate, a general routine analysis and an instrument to solve the research problem.

The gas chromatograph-mass spectrometer is the ideal combination because that is the only way you can handle the nanogram of sample in a mass spectrometer. But the mass spectrometer is also very demanding, because you can't use very dilute solutions, and in most laboratory techniques your handling of one nanogram is no problem. In that respect mass spectrometry has drawbacks, in that you have to have the sample as such. You can't use it in a very high dilution.

Dr. DeGrazia: I can't think of anything easier than freezing it. The patient expires it in the breath, and you collect it frozen.

Chairman Biemann: You are talking about CO₂.

Dr. DeGrazia: Everybody has their own axe to grind.

Chairman Biemann: Those are the two big differences. You either look for something very simple like CO_2 , which doesn't give you any structural information, or you look for the structure of a particular metabolite which comes from a particular compound.

Dr. Berlin: As I sit back and try to translate the experience of those of us who work with carbon-14, into the potentials for the use of carbon-13, the thing that impresses me is the ability to get some structural information. I think maybe we are in the minority here, but I am having trouble in translating this into what we might or might not do in the biological world.

The thing I came here looking for--I almost wrote down two questions: Will carbon-13 replace carbon-14 in the same way we are using it currently? That is one problem. Now you tell me that we are going to derive a lot of structural information from carbon-13. This opens up a whole new avenue, what might be called metabolic biochemical research.

I, for one, am having trouble envisioning how we are going to do that. We hear about a set of instruments, and about the complexity of the mass spectrometer, and then about putting in a computer besides. This is not a simple procedure for those of us who have been accustomed to working with carbon-14. Perhaps we need somebody to give us some rigorous comments as to where we are going and what we are going to be able to achieve, and what the problems are going to be. We have heard some details of problems, but we haven't heard a discussion of the broad aspects of these problems, and I gather we are getting into the area of significant instrumentation problems.

Chairman Biemann: May I take a crack at that question? There are two different problems. One, you can use carbon-13 as one used to use carbon-14, as a general tracer. In that respect there is no question that the method of choice is to burn it to CO_2 and look for the increase in carbon-13 with a compact isotope ratio instrument, just like you used to look for the increase in carbon-14. In that respect those two are similar.

But as far as the structural information is concerned, if you need to have structural information and if you want to know what the compound is that still carries that label, then one should not use any procedure that degrades the structure, but instead use a mass spectrometer with a mass range equal to that of the molecule and with adequate sensitivity. Now one looks at the mass spectrum to find the evidence of label, whether the compound is labeled or unlabeled, and where the label is located. These are two completely different applications, and I would feel that one follows the other. First you find out what you are looking for, and then you determine the structure.

Conversely, the complexity of the instrumentation goes up because in the second application you are really looking more for qualitative information, so the high isotope ratio accuracy is unobtainable. You really don't need it when you have the pure isolated compound. If it

still maintains a few percent of the label, that is enough, as you saw in Dr. Klein's spectra, and you can see that it is there. Then you have to isolate that compound, and for that the gas chromatograph is the thing.

Dr. Wall: Some of you present know that some months ago in my laboratory we had quite a discussion on the exact point Dr. Berlin raised. My group had proposed to try to solve this problem by seeing if there was some way we could work out an automated technique where the sample would be burned to CO_2 . That would all go nicely into the appropriate mass spectrometer, so the idea would be whether you could do carbon-13 plasma the way you do carbon-14 labeled drug and get a $T_{1/2}$.

There was just one flaw in our idea, and that was that there is no instrumentation available, as of now, that would really do it conveniently. But it is my own feeling, that with research and the appropriate effort spent, it is a definite possibility. As you know, when work started with carbon-14 you didn't have nice scintillation counters either. We may be in exactly the same situation now.

The real problem is that with carbon-13 there is always a natural abundance. One of the bugaboos is that this natural abundance may not be absolutely constant, but may vary with the individual, depending on the diet. So, a lot of these things--species, and so on--will have to be worked out. There are those of us who are thinking about it. Quite frankly, the type of sophistication of instrumentation is beyond what I think our group can do. I hope that such an instrument can be developed.

Dr. Hofmann: I may be getting closer to somebody who will make the bridge. It seems to me that if carbon-13 replaced carbon-14 in routine diagnostic use it would not really have any use, because carbon-14 is not used very much in routine diagnostic use. My impression, when I go to rural hospitals, as I sometimes do, is that the kind of counters which are in clinical laboratories are gamma counters, and the reason is two-fold. One is that the gamma isotopes used have short physical lives, and the other is that people are counting body tissues, for which, until recently, it has been difficult to do by liquid scintillation counting.

Secondly, I think for routine diagnostic work we need automated techniques, and many techniques which we are all using routinely, such as chromatographic techniques, have not yet come into routine use because they have not been automated.

It seems certain to me that valuable structural information can be gotten from carbon-13. The problem is that it may be difficult to justify economically. Ultimately, if it leads to advances in the understanding of disease it may be extremely worthwhile economically. I think this is part of the problem.

Dr. Fales: Along that line, I wonder if you could expand a little bit. At the end of your talk today you said you would like to apply carbon-13 labeling to your particular problem. I wonder why you specifically want to. For what reason?

Dr. Hofmann: I am working in clinical gastroenterology and I find that the translation of things I do in the laboratory to routine patient care is a very difficult one. It not only involves all of the problems involved in financing a private clinic, and how the patient pays, but it involves the problem of when you are willing to charge the patient for a test when you are not quite sure what is normal and what is abnormal.

In gastroenterology we are concerned in part with the gut, and we want to know what it can absorb. We would very much like to develop a series of tests based on the principle that we have substances which are not degraded by mammalian enzymes but are degraded by bacterial enzymes. The rate of bacterial degradation is ordinarily slow, with all of the other processes being fast. That is the principle of measuring the rate of bacterial deconjugation, where once the product is cleaved, it goes immediately to CO_2 , but it is not cleaved by mammalian enzymes. Failure to understand this principle, in my judgment, may be responsible for the lack of sensitivity of such things as the lactose tolerances, because carbon-14 lactose when given to normal people goes to the liver and is converted to $^{14}\text{-CO}_2$, and given to people lactase-deficient goes to intestinal bacteria a little later and is converted to $^{14}\text{CO}_2$, and therefore the signal-to-noise ratio is not good.

What we would like to do in our field, we think, is to develop tests, if we can find them, based on bacterial production of $^{13}\text{CO}_2$, and the great advantage for us would be if we can find these we could do the tests right on the floor where the doctor is, without professionally trained personnel and without any worry whatsoever involving the transmission of radioactive samples.

My people at Mayo think this would be desirable. We don't actually have to analyze the sample there. We can take the sample to our laboratory, but we would like to be able to analyze it and get results within several hours, because the doctor wants to know the result that day.

Dr. Fales: It is essentially because it is a health hazard, that carbon-14 is not used more in the clinic?

Dr. Hofmann: Why is carbon-14 not used? I don't think I know the answer. You will have to ask the people in the Division of Nuclear Medicine. I ask them, and they say, "We don't use it because nobody else uses it." I relate this to health hazard and difficulty of counting blood and stool carbon-14.

Dr. Fales: But in your talk you mentioned specifically that the amount of carbon-14 you are giving is no worse than the normal background.

Dr. Hofmann: I really think, as far as this particular breath test is concerned, the only advantage of carbon-13 would be that I can get it out of the laboratory and get it right on the floor and even get it in the home, and get it out into clinics all over Minnesota, where samples can be mailed in if need be. Dr. DeGrazia puts a patient in the helmet. These are field procedures, and we could do 50 tests at once.

Dr. Berlin: You are doing it on trapped samples of CO_2 , and you can do that on samples of carbon-14.

Dr. Klein: You can't take that in the field.

Dr. Tolbert: You need too much carbon-14 to do a decent experiment. With carbon-13 you need much less. For instance, it is quite conceivable that you would have a small tube which would contain a molecular sieve, and at 10-minute intervals for five periods of time after the initial dose has been given the patient would breathe through it for one minute, which would extract the CO_2 very nicely. Then this would be sent back to the home laboratory. Dr. DeGrazia needs a large amount of CO_2 .

Dr. Hofmann: That is wrong. We trap for 3 minutes with 2 mM of base, and we put the solution in the counter and we get the amount of C^{14}O_2 .

Dr. DeGrazia: You have to understand several things about nuclear medicine: 1) it is preoccupied with pictures, and 2) there is a certain amount of expertise which just isn't available in every nuclear medicine department for isolating materials in a laboratory. People are not specifically trained for this, and many of the nuclear medicine divisions are run by radiologists and not by people trained in nuclear medicine.

Dr. Klein: I want to refer back to Dr. Nyhan because one of the things he pointed out is that you can't do control studies in children. This is one of the big problem areas. You cannot use any carbon-14 unless it is a very, very unusual circumstance.

Dr. Berlin: That is an entirely different problem.

Dr. Nyhan: I think the issue is, to some extent, a psychological one. I think it is true that those of you who do calculations (and I do them too) can reassure yourselves that the amount of radiation you are using is miniscule. The doses that we use for carbon-14 in people come out to the dose you get with the chest x-ray.

The point is, nobody really knows what is happening to the chromosomes when you get these radioactive compounds incorporated into the nucleic acids. People don't really know. We can look under the microscope, for instance, and see cells that have tritiated thymidine in them, and can see the chromosome breaks. And a few years ago we didn't know about such damage because we didn't see the breaks. I think people are going to be less and less reassured by all these calculations, and clinicians are not going to be able to use carbon-14.

Dr. Hurwitz: The problem is the therapeutic orphan, the child who doesn't have a long life expectancy, or the pregnant woman. Or, if you want to study the placental transfer of other compounds, how are we going to deal with these people with the standard radioactive isotopes we have today? In therapeutics we don't know how to treat these patients. The PDR always says, "Not tested in children or pregnant women." We are going to have to be able to do them by non-radioactive methods.

Dr. Mirkin: There are two points. The National Research Council recently held a meeting directing themselves to several points which were brought up here. First, radioactivity has been administered to two normal children in studies which cleared the human volunteer committees at several major institutions in the Midwest and in the East. Whether this is a recommended or desirable procedure is still a moot point.

With regard to pharmacologic agents which may not be cleaved into components which enter into rapidly developing cells, I think this perhaps may be an acceptable step, as pointed out by Dr. DeGrazia. The half-life of these compounds is relatively brief, and this may be fine. However, when you get into the realm of the pregnant female, how can you really and truly justify radioactive compounds?

We have just been doing this now on border specimens, and we are beginning to get the initial data on drug distribution in the fetus. It is astonishing that drugs do persist in very unexpected areas. I would submit that perhaps this technology is not applicable to that particular kind of problem, and that we should think again that there is a dichotomy of problems to which our technology is applicable with respect to radioactive compounds.

In those situations where we want rapid measurements of drugs, to relate them to pharmacologic and therapeutic effects, I am not sure this technology is clearly applicable. I would prefer radio-immune assay. I would prefer the gas chromatographic procedures which we are using routinely. I think when we want the structural compounds here we should use this technology for the kind of information which is not readily available, and this is essentially what I wanted to have some comment on--whether or not we are trying to over-extend the application of stable isotopes at this meeting.

Chairman Biemann: Since this is a discussion on techniques and instrumentation, perhaps we should stay away from the pregnant woman a little bit (Laughter) and concentrate on the technique aspects.

Dr. McInteer: I have some results on the CO₂ analysis that I would like to present. This was a Monday afternoon experiment that we did, the results are sufficiently encouraging to present them to you.

A ^{13}C EXPERIMENT USING MASS SPECTROMETER BREATH ANALYSIS

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As a prototype to the use of ^{13}C for clinical, medical, or other biological research, an experiment has been run (November 1, 1971) in which the carbon-dioxide of breath samples is analyzed for carbon isotope abundance following ingestion of carbonated water with enriched ^{13}C .

Drinking water was carbonated with 45 psi of carbon dioxide of 90% ^{13}C at 10°C . A 100 ml portion was drunk containing 1.34 g CO_2 , corresponding to 0.35 g ^{13}C . Breath samples were collected in 50 ml evacuated bulbs and the carbon dioxide was isolated cryogenically as follows: First, the entire sample was chilled with liquid nitrogen and all non-condensables were removed by pumping. The mass-28 peak of the bulb contents was observed directly by the mass spectrometer to verify complete-removal of these gases. The bulb was then allowed to warm to -90°C at which temperature the carbon-dioxide was released, typically reaching 10 to 12 mm pressure. A portion of this gas was analyzed for the mass 44, 45, and 46 peaks on a Model 21-620A mass spectrometer, manufactured by Consolidated Electrodynamics Corporation, Pasadena, California. Total time for processing and analyzing a sample was about ten minutes.

Results are displayed in Figures 1 and 2 in terms of the percent contribution of the mass-45 peak to the total CO_2 peaks. Reference samples taken immediately before and well after the test gave identical values, 1.154%, although previous experience indicates that variations of 0.004% can occur. The data of Figure 2 are expressed as the excess above this reference value.

Although the purpose of this experiment does not presume to include interpretation of the physiological processes which are involved in the fate of CO_2 in seltzer water, several details seem obvious. The initial high value dropping rapidly is due to the enriched CO_2 entering the lungs directly during the time of drinking the sample. This is a highly carbonated solution and quite bubbly. The normal ventilation of the lungs dilutes this initial dose rapidly (about a one minute time constant). The odd point at ten minutes is a burp. By about this time the processes conveying CO_2 from the stomach to the blood stream to the lungs has started to show up, reaching a maximum

at 23 minutes and then dying away. By 300 minutes there is no detectable excess ^{13}C in the breath.

In summary, it has been shown that the technique of analyzing the isotopic ratio of expired carbon dioxide following ingestion or injection of ^{13}C labeled compounds may be made simply, rapidly, accurately, and sensitively. It should be emphasized that the mass spectrometer used for this experiment is *not* specially designed. To the contrary, it is normally used for chemical process control and is subjected to widely varying isotopic compositions of many different gases. There is every reason to believe that improvements of sensitivity to variations from normal abundance are possible to the extent of a factor of 10 to 100. Sample taking and handling should be open to considerable simplification and speed-up. Finally, it has been demonstrated that breath analysis shows no "biological noise" so that curves such as those of Figure 2 are susceptible to accurate quantification and analysis. This is the first demonstration of such techniques.

The author is indebted to Joe G. Montoya for mechanical and analytical assistance.

Figure 1. Percent ^{13}C in breath samples following ingestion of 100 ml carbonated water containing 0.35 g ^{13}C .

Figure 2. Percent ^{13}C in breath samples following ingestion of 100 ml carbonated water containing 0.35 g ^{13}C .

DISCUSSION

Dr. Wall: Will you explain again the background curve?

Dr. McInteer: Background data was taken just before and after the test. It is the normal abundance that is present. I have been unable to detect any variation in this abundance either between individuals or in a single individual at different times of the day.

Dr. Chidsey: How do you explain the second peak?

Dr. McInteer: The first peak is the result of the lungs being emptied. Meanwhile, whatever be the physiology involved in transporting enriched carbon from CO_2 to the blood stream into the lungs and then through the breath, it apparently takes 20 minutes to appear.

Dr. Chidsey: The recirculation wouldn't take that long. Do you mean the individual as he drank the CO_2 got some in his breath and inhaled it?

Dr. McInteer: Yes.

Dr. Dayton: What are the educational requirements of the person who is operating the instrument in your laboratory? What are the space requirements? What are the service costs of the instruments involved?

Dr. McInteer: The person who was operating the instrument in the laboratory was a high school graduate. The instrument, which incidentally happens to be the model No. 620A, made by Consolidated Electrodynamics Corporation, seems to require a bit of routine maintenance maybe every six months. As to the space, a table size sort of thing. There is a recorder.

Dr. Borg: Are we talking about the dilution ratio of 10^4 and 10^5 ? Do you expect that from this machine?

Dr. McInteer: No. Its demonstrated ability to measure was $\pm 0.004\%$.

Chairman Biemann: 350 mg of 90% labeled CO_2 is quite a slug. A dose that is one magnitude less gets you down into your limit of error.

Dr. McInteer: The limit of error of 0.004% is simply the capability of reading from a normal recorder chart. These data are really more consistent than my preliminary experiments would have indicated.

Dr. Watson: I have a question based on the precision that accompanies the high sensitivity with low levels of detection, which is not necessarily directed to Dr. McInteer. To set the scene, in our laboratories at Vanderbilt we are working with prostaglandin and PGF₂, and we use a deuterium labeled species comparable to that shown by Dr. Sweeley. We can measure on the order of 100 pg of this material, but we cannot do it with much better precision than $\pm 20\%$.

I am a little concerned about why we can't do better. Perhaps it is just my own statistics. Dr. Klein, you mentioned that you can see one part in 500, but you need 5 μ g roughly. That means that the less abundant species is present at a level of 10 ng. Is that always the case? If you want to measure one part in 10,000, do you still need 5 ng of the less abundant and 50 μ g of the more abundant species?

Dr. Klein: I don't know; we measured one part in 10,000 with the double-labeled barbiturate. I think the student who is doing it was using perhaps 10-20 μ g.

Dr. Watson: So you still need the order of 5 ng at the low end.

Dr. Klein: I haven't calculated what that comes out to, but I think there is a real problem in terms of ultimate detection when one considers the actual amounts of sample you are putting in. The ratios themselves are not limiting.

Dr. Sweeley: I think it is the noise of the system that was limiting you in terms of accuracy at the lower level. In the experiments I was doing, I was pushing the machine as hard as I could. The minor component was pretty bad.

Dr. Watson: What precision do you find?

Dr. Sweeley: You saw the plot. The standard error was absolutely horrendous. I didn't point that out.

Dr. Watson: That was 1 ng.

Dr. Sweeley: We have gone down to about 100 to 200 pg of prostaglandins, and again the precision is not so hot. We are trying to do an injection of sub-microgram amounts.

Chairman Biemann: That is one of the things we have to keep in mind also. There is a difference in determining the labeling of that material and whether you want to have a quantitative measure of how much is there.

Dr. Watson: Something mentioned earlier was a bit misleading to me, and that was that the sensitivity increases in direct proportion to the number of labels included.

Dr. Klein: No. As the exponent of the number of labels.

Dr. Watson: Doesn't that mean your background is suppressed in proportion to the increased sensitivity?

Dr. Klein: True, because you are moving over to mass numbers where there is less and less natural isotopic contribution. But as even in the tetradeuterio bile acid when you go out to that $m+4$ ion, where you should have very little background, you get much more than you would expect and it appears to come from the spectrometer itself. That is what really does limit you.

Dr. Watson: That is my point. I thought someone said that the sensitivity increases, when in fact the background decreases to a reasonable level, and not, ultimately, down to nothing.

Dr. Tarlov: I would like to say that I think one of the very severe restrictions upon clinical investigation at the present time is the public reluctance to accept radioactive isotopes in normal individuals. This has been alluded to here before. I think one of the great challenges for the scientific community in the next couple of years is going to revolve around toxicity studies which are going to give the FDA and the public confidence that these stable isotopes are safe. I wonder what information is available on that subject.

Dr. Sweeley: May I show a couple of slides on a metabolic study utilizing deuterium-labeled glucose? I think it represents a useful example of the application of stable isotopes in human metabolism experiments.

AN *IN VIVO* STUDY OF THE METABOLISM OF HUMAN PLASMA GLYCOSPHINGOLIPIDS
USING 6,6 DIDEUTERIOGLUCOSE AS A TRACER

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Without a lot of preamble, there exists a group of inherited lysosomal diseases of sphingolipid metabolism, which are based on the deficiency of specific glycosyl hydrolases and other types of hydrolases. One such genetic disorder is Fabry's disease, in which there is a slow progressive accumulation of a ceramide trihexoside, galactosyl ($\alpha 1 \rightarrow 4$) galactosyl($\beta 1 \rightarrow 4$)glucosyl ceramide (Figure 1), in most of the organs in the body, particularly blood vessel walls and kidney. The ultimate consequence of this lipid accumulation is usually death from renal failure. One of the problems has been to determine what the major precursors of this lipid are and from what principal source(s) the lipid and its precursor is derived.

In the plasma of these patients one can detect an elevation in the concentration of the ceramide trihexoside to about three times normal levels. That is, the normal level is about 2 $\mu\text{g}/\text{ml}$ whereas the level in a Fabry patient is about 6 $\mu\text{g}/\text{ml}$. A former student in my laboratory, Dr. Dennis Vance, wanted to find out particularly whether red cell membrane globoside, which is a closely related glycosphingolipid with a terminal aminosugar moiety attached to the ceramide trihexoside structure, might be a precursor of the extra circulating ceramide trihexoside in Fabry patients. We considered the possibility of conducting an experiment *in vivo* with radioactively labeled glucose but came to the conclusion that it really would not be possible with 100 μCi (the upper limit of ^{14}C , that, I believe, could be administered at the time this experiment was planned a few years ago). Considering that 10 ml aliquots of plasma would be obtained for one analysis, about 20 μg of the ceramide trihexoside would be obtained from individual samples, representing about 4 μg of isolated glucose. Assuming that 50 dpm above background would be a minimum level for adequate counting statistics, the specific activity of the glucose would have to be on the order of at least 12,000 dpm per milligram to carry out the study. We estimated that this would require injections of millicuries rather than microcuries of glucose, and therefore turned our attention to the possibility of utilizing a stable isotope-labeled form of glucose as a substrate.

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Although d₇-glucose was already commercially available from Merck, Sharpe and Dolme (Montreal), we were worried about the possibility of biological isotope effects with this compound, and therefore ordered a custom synthesis of 6,6-d₂-glucose after first determining that the mass spectral behavior of this compound would be suitable.

Shown below is Steele's equation for determining the specific activity of plasma glucose that might be obtained after infusion of

$$\frac{SA_t}{100} = \frac{F}{g} + (P/c_o - F/g)e^{-gt/c_o}$$

radioactive glucose (1). We used the same equation to predict what level of d₂-glucose might be obtained in the plasma pool after infusion of a large amount of the labeled compound. The terms in this equation are as follows: g is the inflow/outflow rate of glucose from plasma in grams per hour, P is the priming dose of d₂-glucose in grams (injected at the start of the experiment), t is the time in hours after time zero, c_o is the glucose pool in the body in grams, and F is the infusion rate in grams of d₂-glucose per hour. Assuming the body glucose pool is 0.284 grams per kilogram of body weight and an inflow rate in plasma of 3.8 grams of glucose/m²/hour, it was predicted that if 6,6-d₂-glucose were infused at a rate of about 3 grams per hour after a priming dose of 5 grams, it might be possible to attain a level of about 30% of the body glucose pool as labeled species (2).

The methyl glycoside tetraacetate of 6,6-d₂-glucose had the mass spectrum shown in Figure 2, which is similar to that of the protium form established by DeJohng and Biemann some years ago. After comparing the spectra of the labeled and unlabeled species we concluded that determination of the intensities of m/e 200 and 202 would enable us to monitor for the amount of labeled glucose incorporated into glycosphingolipids and to determine their fractional turnover rates in plasma.

The study was carried out with a medical student volunteer and a patient with classical symptoms of Fabry's disease and typically high levels of ceramide trihexoside in his plasma. Blood samples were taken at various times from one hour to 140 days after the infusion began, and the levels of 6,6-d₂-glucose in various plasma and red cell glycosphingolipids were determined.

The gas chromatographic separation of the methyl glycoside tetraacetates of glucose and galactose is illustrated in Figure 3. The single peaks obtained for each of the sugars were nearly completely separated even with retention times of about 10 minutes, making it possible to carry out about 3 analyses per hour. As previously

observed in the gas chromatography of stable isotope-labeled carbohydrates, there was a chromatographic isotope effect which caused partial resolution of the protium and 6,6-d₂-glucose species. This effect is shown clearly in an analysis of the composition of a blood glucose sample obtained about 5 hours after the infusion began (Figure 4). Manual integration of the areas of the curves for m/e 200 and 202 and background subtraction of the blank intensities indicated that the plateau level of labeled glucose was indeed about the 30% which had been calculated from Steele's equation.

Figure 5 shows the results obtained for the incorporation of labeled glucose into the plasma pool of glucocerebroside, comparing the patient (A.G.) and the control (L.S.). Reasonably good curves were observed, with maximum incorporation of the 6,6-d₂-glucose at approximately 48 hours after the start of the infusion. The curve from 72 hours to later times gave semilogarithmic plots from which fractional turnover rates and turnover times could be calculated. I am not going to show the corresponding curves for the plasma pool of ceramide trihexoside because it would take too long to explain the results obtained, but I can tell you that the data supported the hypothesis that the ceramide trihexoside was derived mainly from red cell membrane rather than from liver synthesis.

It is not likely that this experiment could have been carried out in another way. It was a tedious study since it required about 6 months for Dr. Vance to complete the analyses of all of the plasmas and red cell fractions. This does not lessen the value of the experimental approach, however. I think it amplifies what Dr. Biemann said, that for an experiment with only a few subjects, where an attempt is made to answer specific questions, the stable isotope approach might be superior to any other method. The cost of the metabolic study I have described was not out of line with radioactive tracer studies. The labeled glucose cost approximately \$1,000 for 100 grams of purified compound, a solution of which was sterilized and shown to be pyrogen-free prior to use.

REFERENCES

1. Steele, R, Wall, J. S., DeBodo, R. C., and Altszuler, N. *Amer. J. Physiol.*, 187: 15 (1956).
2. Vance, E., Ph.D. Thesis, University of Pittsburgh, (1968).

Figure 1. Partial structure of the ceramide trihexoside that accumulates in patients with Fabry's disease.

Figure 2. Mass spectrum of methyl 2,3,4,6-tetraacetyl-6,6-²H₂-glucopyranoside (D. E. Vance, Ph.D. Thesis).

Figure 3. Gas chromatographic separation of the methyl glycoside tetraacetates of glucose and galactose and mannitol hexaacetate of 8% Apiezon L coated on 80-100 mesh Gas Chrom Q, 200° (D. E. Vance, Ph.D. Thesis).

Figure 4. AVA traces which show the intensities of m/e 200 and 202 during elution of a mixture of protium and d_2 forms of methyl glucoside tetraacetate derived from a plasma glucose sample (D. E. Vance, Ph.D. Thesis).

Figure 5. Incorporation of 6,6- d_2 -glucose into plasma pools of glucocerebroside in a normal control (L.S.) and a patient with Fabry's disease (A.G.) [D. E. Vance, Ph.D. Thesis].

DISCUSSION

Chairman Biemann: Can that much glucose be tolerated without ill effect?

Dr. Sweeley: We saw no evidence of ill effects over the course of the experiment.

Chairman Biemann: We haven't heard any comments from Dr. Gaffney or Dr. Horning.

Dr. Horning: Perhaps one point was brought up earlier. The dilution procedure is really directed to a simple analysis of the concentration, and I am not at all sure the mass spectrometer in its present form is going to be the best way to do this. One might need much greater sensitivity in the case of prostaglandins, which we are concerned about, as Dr. Watson is.

Dr. Watson: A point there. One does need in some cases, you see, a carrier to minimize the adsorption of the endogenous material

Chairman Biemann: Time is running out, gentlemen. I see Dr. Gaffney in the back of the room and wonder if he has any comments to make on this subject.

Dr. Gaffney: Just one or two points... If there is any way to get reliable quantitation besides quantitative GC/MS, it is probably to be preferred at this point in time. I have this impression despite the fact that, as shown in Figure 1, you can see as little as 50 pg each of drug and stable isotope-labelled internal standard using the LKB 9000 GC MS. As shown in panel B you can do isotope ratios on 50 pg of trifluoro-acetylated deuterated and non-deuterated nortriptyline. Mass 232 is the base peak of TFA nortriptyline, whereas mass 234 is the base peak of dideuterium-labeled TFA nortriptyline.

I have one other comment; it comes back to Dr. Berlin's point about molecular specificity. I think the price that the pharmacologist is willing to pay for molecular specificity should be a very, very high one.

Chairman Biemann: With this note we will have a brief break.

Figure 1. Base peak fragmentograms of 100 picograms (A) and 50 picograms (B) of nortriptyline-trifluoroacetate (m/e 232), deuterated NT-TFA (m/e 234), and IBD-18 (m/e 238) (C) solvent injection.

INSTRUMENTATION - NUCLEAR MAGNETIC RESONANCE

Chairman: J. Goldstein

NUCLEAR MAGNETIC RESONANCE

N. A. Matwiyoff

SOME APPLICATIONS OF ^{13}C NMR TO BIOLOGICAL PROBLEMS, PAST AND FUTURE

Charles T. Gregg

NUCLEAR MAGNETIC RESONANCE

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It is apparent from the discussion this morning that Los Alamos Scientific Laboratory has a substantial and continuing interest in the separation of the stable isotopes of carbon, nitrogen, and oxygen (ICONS), and in their conversion into chemically useful forms. But I would like to emphasize here that that is only one aspect of a larger ICONS program, the objective of which is to develop the utility of stable isotopes in basic research and applied studies.

As a consequence, we have a number of active and growing studies in a variety of areas, including organic chemistry, biology, biochemistry and chemical physics. Due to the time it took us to develop a group at Los Alamos, equipped to proceed on a relatively broad front with stable isotopes, we can now appreciate some of the problems you might have in setting up your own programs. We invite you to call on us. We are happy to discuss the problems we have had, share the information we have acquired with groups in other disciplines, and invite you to do the same. Take advantage of the unique national resource in stable isotopes that we have at Los Alamos.

Before I start on the topic for discussion, I would like to emphasize a point that Drs. Berlin, Mirkin, and Gaffney made during the last instrumentation talk. We are not here to demonstrate that ^{13}C can replace ^{14}C in clinical chemistry.

Carbon-13 and the other stable isotopes should be considered on their own merits. They can be used as non-radioactive tracers, but they can also provide a great deal of structural information. This afternoon I would like to talk about nuclear magnetic resonance spectroscopy (NMR) as applied to isotopically-labeled molecules.

Although NMR is usually thought of as a structural technique, it can also be used for routine analytical measurements. At the present time, due to the cost and sophistication of ^{13}C instrumentation, the major applications of ^{13}C NMR have been to structural problems. Some important features of ^{13}C NMR as they might apply to the research aspects of clinical chemistry are summarized in Table I. Nuclear magnetic resonance spectroscopy can be used to determine not only the presence but the location of a ^{13}C atom in a molecule. With ^{13}C NMR, such high resolution can be obtained that each individual atom in the molecule can be indentified; thus, each molecule usually has its own particular molecular signature and, as a consequence, analysis of complex mixtures is very often possible. A potential application in clinical chemistry would be the determination of the clearance of ^{13}C -labeled urea from blood after an intravenous injection. Despite the fact that there are many carbonyl compounds in blood serum or urine, the ^{13}C NMR spectrum of urea is unique.

One could also use, for example, specifically-labeled alanine *in vivo* and determine the redistribution of the ^{13}C label in that compound and the transfer of label to other metabolites. I would re-emphasize, both from the analysis point of view and from the structural point of view, that quantitative NMR measurements on crude mixtures is also sometimes possible.

The minimum sample size is about 0.2 micromole of ^{13}C , which makes it a relatively insensitive tracer by comparison to mass spectrometry. However, in any dose-loading experiment, this is an attainable sample size. The detectable dilution for compounds which have at least two adjacent ^{13}C atoms in them is about one part in 10^4 . Don't be alarmed at the instrument cost requirements listed in Table I. There exists a wide range of spectrometers, from both the point of view of their sophistication as well as their cost. Many of the examples I will give could have been analyzed with very simple proton spectrometers which cost from \$8,000 to \$35,000. I would like to concentrate on the ^{13}C NMR techniques using the most sophisticated spectrometers with cost ranging from \$80,000 to \$250,000.

The sophisticated instruments that I am going to talk about are widely distributed throughout the country. Many NMR spectroscopists, all over the country, are eager to collaborate with biologists.

The magnetic resonance technique utilizes the fact that isotopic nuclei, such as ^{13}C , ^1H , and ^{15}N have unique nuclear spin magnetic moments. The magnetic moment confers on the nucleus the property of having a non-classical dipole when the nucleus is placed in the magnetic field. This is indicated schematically in Figure 1 where the static magnetic field is represented by the arrow labeled H_0 . Because of its microscopic bar magnet properties the ^{13}C nucleus can take one of the two orientations in the magnetic field shown in Figure 1, an orientation aligned with the field or one opposed to it. The orientation denoted by $m_I = 1/2$ is a low energy state and the other is a high-energy state populated via thermal processes in the liquid.

The energy (E) of the oriented nucleus in the magnetic field is given by the product of the magnetic field strength (H_0) and the nuclear magnetogyric ratio (γ). The ratio (γ) is a parameter which is characteristic of a given nucleus and is, for example, quite different for ^{13}C and ^1H . Thus, the energy difference between two states measured by the NMR spectrometer is a product of the nuclear parameters and the magnetic field strength. Through well-known relationships this energy difference can be expressed in units of kilocalories (ΔE), units of field strength (H_0 , gauss or kilogauss) and frequency units (ν , cycles or Hertz and ω , radians). The relevant equations for these conversions are illustrated in Figure 1 where $\hbar$ represents Planck's constant h divided by 2π . This is the static picture. When the dipole is placed in the magnetic field it is not static but precesses about the field direction (as shown in Figure 1) at a characteristic frequency, called the Larmor frequency, which can be shown to be the frequency ω or ν of Figure 1.

Experimentally the magnetic dipoles are subjected to a magnetic field in which they are polarized. By use of an electromagnetic field from a radiofrequency transmitter, transitions are induced between the two polarized states. In the process, energy is transferred between the spin system and the electromagnetic field, and this resonance transfer of energy is picked up via a sensitive detector. The condition for such resonance is that the electromagnetic field is oscillating at exactly the Larmor frequency. Because each atomic nucleus has a unique magnetogyric ratio γ , the conditions for resonance differ greatly for each nuclide. At the bottom of Figure 1, the startling difference in the nuclear resonance frequencies of ^{13}C (25.2 MHz) and ^1H (100 MHz) is listed for a static magnetic field of 23 kilogauss.

The difference, in the magnetogyric ratios, although large, would be merely a curiosity to the physicist were it not for the ability to resolve resonance frequencies at high resolution; modern electronics allow us to distinguish radio-frequency signals separated by as little as 0.1 Hz. At high resolution, more than one signal can be observed for each nuclide. The resonance seen depends not only on the nuclide *per se* but on its site within a molecule. This effect on the parameters for resonance is referred to as chemical shift.

The chemical shift of the resonance frequency of a nucleus such as ^{13}C has its origin in the electron density surrounding the nucleus in a molecule. As depicted in Figure 2 the electron density surrounding the nucleus is forced to undergo precession in the magnetic field. This forced precession, like the flow of electrical current in a closed loop, induces a magnetic field at the nucleus, thereby shifting the value of the radio-frequency oscillating field at which the nuclei undergo resonance. These chemical shifts are quite large for ^{13}C and closely correlated with the charge density at the carbon atom--in general, the greater the charge density at the carbon atom, the higher the value of the applied field at which the nucleus undergoes resonance for a fixed frequency.

In Figure 3 the ^{13}C spectrum of glycine is depicted. Along the ordinate is plotted the intensity of resonance (proportional to the number of ^{13}C spins) and along the abscissa is the value of the applied field in gauss or in Hz, as interrelated through the equation $\hbar\omega = \gamma H$. As expected, the $^{-13}\text{CH}_2$ -carbon which carries a larger electron density than the ^{13}C atom in $^{-13}\text{CH}_2$, undergoes resonance at a higher degree of the applied field. Another feature to note is the high degree of resolution apparent in the spectrum--the chemical shift is 4000 Hz and the line widths are 1-3 Hz. Because of the large shifts, each chemically distinct ^{13}C atom in a molecule makes a unique contribution to the NMR spectrum even in a complex molecule like chlorophyll b as depicted in Figure 4. Despite the complexity of the chlorophyll molecule, we have been able to assign the resonances and correlate the chemical shifts with the atom charge densities with each ^{13}C atom in the molecule. For example, the carboxyl and carbonyl resonances of the chlorophyll molecule occur at the extreme left end of the spectrum (low field) whereas the electron-rich alkyl carbon resonances occur at the highest fields (at the extreme right end of the spectrum).

Neighboring magnetic dipoles can add to or subtract from the applied field, splitting the line into a multiplet. As depicted in Figure 5, structural information can be deduced from this coupling of nuclear dipoles. The character of the multiplet (as determined by the spacing between the lines, and whether it is a doublet, triplet, etc.) the intensity distribution is directly related to the kind and number of nuclear dipoles interacting with the nucleus under observation and to their distance from it. An example of this coupling is shown in Figure 6 where a spectrum of 90% ^{13}C -labeled acetic acid is reproduced. In the carboxyl region of the spectrum, the quartets are due to coupling of $^{-13}\text{CO}_2\text{H}$ with the remote protons of the $-\text{CH}_3$ group. The doublet nature of the quartets arises from those molecules (~80% of the total) which contain adjacent ^{13}C atoms, and the central quartet is due to the ~10% of the molecule in which the $^{-13}\text{CO}_2\text{H}$ group is adjacent to a ^{12}C atom which lacks a nuclear dipole moment. The same sort of pattern is apparent for the $-\text{CH}_3$ group. However, the spacing between the lines is greater because the ^1H dipoles are directly bonded to the ^{13}C atom. Figure 7 is the spectrum of glycine enriched to the 90% level with ^{13}C . The multiplet patterns are now doublets (for the ^{13}C - ^{13}C coupling) of triplets (for the coupling of ^{13}C with two ^1H dipoles).

To summarize, the ^{13}C NMR spectrum is determined by chemical shifts and the multiplet pattern. These two components of the spectrum are often characteristic enough that complex mixtures can be analyzed. In particularly complex situations, one can also resort to measurement of ^{13}C relaxation times but that subject would require a separate discussion. Instead I will merely point out that ^{13}C NMR line widths are a function of the rate at which the excited spin states are relaxed (by molecular motions) into the lower energy state and of the chemical environment of the ^{13}C . Note the difference in the line widths associated with the $^{-13}\text{CH}_2$ and $^{-13}\text{CO}_2\text{H}$ carbon atoms in glycine (Figure 3). It should be

noted that the spectra in Figures 3 and 4 were obtained under conditions such that the proton couplings with ^{13}C were removed electronically. The multiplet patterns which are obvious in the spectrum of chlorophyll (Figure 4) are due to ^{13}C - ^{13}C coupling in the molecule which has been enriched to the 90% level with ^{13}C .

Carbon-13 NMR spectroscopy can be used for quantitative analysis. The ^{13}C NMR spectrum of doubly-labeled acetic acid is shown in Figure 8. This is a proton-decoupled spectrum and, as mentioned before, is accomplished electronically by irradiating the proton spins with a strong magnetic field at their Larmor frequencies while observing the ^{13}C resonances. What is then observed is a doublet in the carboxyl region of the spectrum due to splitting of the $^{-13}\text{CO}_2\text{H}$ resonance line by the ^{13}C in the methyl group. The same thing happens in the methyl group due to splitting by the ^{13}C in the carboxyl group. The intensity of the carboxyl doublet relative to the intensity of the singlet is directly related to the relative concentrations of ^{13}C and ^{12}C in the methyl group. The same is true of the relative distributions of the intensities of the lines in the methyl group resonance.

Carbon-13 labeled β -sitosterol has been used in steroid metabolism studies. It was of interest to determine the ^{13}C content of the product and to assess the extent to which the synthetic procedure had labeled the compound at C-3 and C-4. This material was synthesized by Malcolm Thompson of the USDA. It was prepared in a multi-step reaction sequence involving an Oppenauer oxidation of natural β -sitosterol followed by the replacement of the 3 and 4 carbons of the molecule (Figure 9) by the ^{13}C -methyl and ^{13}C -carboxyl carbon atoms of labeled phenyl acetate. Figure 9 is the proton-decoupled ^{13}C NMR spectrum of the labeled β -sitosterol.

On the basis of chemical shifts alone, using a set of self-consistent substituent parameters, we can predict that the upper signal in the figure is due to C-3 (low field) and the lower resonance (high field) is due to C-4. Moreover, in β -sitosterol, C-3 is directly attached to one proton and C-4 to two. In the proton-coupled spectrum, each component of the C-3 signal should split into a 1:1 doublet, whereas the C-4 resonances should split into 1:2:1 triplets, also confirming these spectral assignments. From the intensity distribution within the ^{13}C - ^{13}C multiplets in the proton decoupled spectrum, the ^{13}C concentrations listed in Figure 9 were calculated. The relative distribution of intensities observed is a reasonable one on the basis of the expected reaction mechanisms.

The accuracy can be improved considerably if there is less than 90% ^{13}C at one position. The spectrum shown in Figure 10 is a proton-decoupled spectrum of acetic acid containing different amounts of ^{13}C labeled in the carboxyl and methyl groups. This is reflected quantitatively in the relative intensity distributions within the ^{13}C - ^{13}C multiplets. The abundance figures listed were derived from those relative intensities.

For demonstration purposes, we have added to human blood serum and human urine micromolar amounts (per ml of sample) of acetate and glycine doubly labeled with ^{13}C . Despite the large background arising from the organic molecules containing natural abundance ^{13}C in these fluids, the labeled substance is readily detectable because of the large chemical shift effects and the easily recognizable ^{13}C - ^{13}C spin-spin coupling patterns of the multiply-labeled tracers. We recognize that micromolar amounts of tracer may be out of the question for routine clinical tests. ^{13}C NMR spectroscopy may not provide a routine clinical analysis procedure but the opportunity it provides for the analysis of each component of a complex mixture in intact biological fluids does provide a new context in which clinical research experiments can be conducted. The analysis of an unfractionated, unpurified biological fluid in a tracer experiment could provide the background necessary for the design of routine clinical assays by a technique that is simpler and less expensive than other spectroscopic methods.

Blood samples can be directly analyzed by ^{13}C NMR spectroscopy. As shown in Figure 11, ^{13}C NMR spectra can be obtained for human blood (upper portion) and human blood subjected to a ^{13}CO atmosphere. The "new" ^{13}C signal which appears in the low field region of the ^{13}C NMR spectrum of carbonylated (with ^{13}CO) hemoglobin can be assigned to the carbon atom of the CO moiety of carbonyl hemoglobin. The chemical shift and shape of the ^{13}C resonance of carbonyl hemoglobin in the intact red blood cell has in it implications regarding the structure of the CO-hemoglobin complex, especially when comparisons are made to the ^{13}C resonances of model Fe(II)-CO complexes. While the details of these spectra have not been fully assessed, the experiments dramatically show that the ^{13}C tracers can be easily seen in complex biological mixtures by NMR spectroscopy.

TABLE I
CARBON-13 NMR AND CLINICAL CHEMISTRY

ANALYTICAL METHOD: Each ^{13}C atom in a distinct chemical site gives rise to an NMR signal whose intensity is proportional to its concentration.

POTENTIAL APPLICATIONS: Rate of clearance of ^{13}C -labeled compounds. Redistribution of label in specifically labeled compounds. Abnormal metabolites in urine or blood. Analysis of crude mixtures possible.

SAMPLE REQUIREMENTS: Minimum Sample Size -- 0.2 micromoles of contained carbon-13.

INSTRUMENTS: Proton or carbon-13 NMR spectrometers widely distributed--Cost \$8K - \$250K.

BASIC NMR EXPERIMENT

^{13}C AND ^1H NUCLEI BEHAVE AS MICROSCOPIC "BAR MAGNETS" IN A MAGNETIC FIELD.

$$E = -\gamma \hbar H_0 m_I$$

$$\Delta E = \gamma \hbar H_0$$

$$\omega = \gamma H_0$$

$$\omega = 2\pi\nu$$

ν FOR ^{13}C IS ~ 25.2 MHz FOR
 $H_0 \approx 23$ KG.

ν FOR ^1H IS ~ 100 MHz FOR
 $H_0 = 23$ KG.

Figure 1. Schematic of the basic nuclear magnetic resonance experiment.

DIAMAGNETIC SHIELDING OF THE
NUCLEUS OF AN ISOLATED ATOM.

Figure 2. Origin of the chemical shift of the nuclear resonance.

Figure 3. Proton noise-decoupled ^{13}C NMR spectrum of glycine.

Figure 4. Proton noise-decoupled ^{13}C NMR spectrum of chlorophyll b enriched to the 90% ^{13}C level.

STRUCTURAL INFORMATION IN THE COUPLING OF NUCLEAR DIPOLES

Figure 5. Origin of nuclear spin-spin coupling multiplet patterns in NMR spectra.

Figure 6. ^{13}C NMR spectrum of 90% ^{13}C -labeled acetic acid.

Figure 7. ^{13}C NMR spectrum of 90% ^{13}C -labeled glycine.

PROTON NOISE DECOUPLED ^{13}C NMR SPECTRUM
OF ACETIC ACID - 1, 2 ^{13}C (93 %)

Figure 8. Proton noise-decoupled ^{13}C NMR spectrum of 90% ^{13}C labeled acetic acid.

Figure 9. Proton noise-decoupled ^{13}C NMR spectrum of β -sitosterol in the region of the C-3 and C-4 resonances.

PROTON NOISE DECOUPLED ^{13}C NMR SPECTRUM
OF ACETIC ACID -1,2 ^{13}C

Figure 10. Proton noise-decoupled ^{13}C NMR spectrum of ^{13}C -labeled acetic acid.

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Figure 11. Proton noise-decoupled ^{13}C NMR spectra of human whole blood (a) and human whole blood carbonylated with ^{13}CO .

DISCUSSION

Chairman Goldstein: I would like to add just one comment here-- that all of the preceding talk revolved around labeled carbon-13 materials, but not everyone has access to the labeled materials that Matwiyoff does. So, most of the people are working either with very simply labeled materials or, in a great majority of cases, with carbon-13 in natural abundance.

For example, we have finished a study of an antibiotic with 22 different carbon atoms, each one of which gives a distinct signal that can be uniquely and definitely assigned to a position in the antibiotic. This indicates that something can be done without having the superb refinement of the labeling facilities that we have heard described here.

Now we have a panel available to respond to your questions. I would like to urge you to raise for this panel, in light of the previous talk, whatever questions regarding instrumentation and techniques might appeal to you.

Dr. Kipnis: The biochemist has very tedious techniques for breaking down the glucose molecule to its individual carbon atoms and determining their isotope content. It seems to me this technique ought to be very useful in doing this with carbon-13.

I would like to know how much you need. For example, if one were to have the proper labeled precursor, (and from the biochemist point of view we know what the precursor would be), give that compound to someone and isolate the glucose, one could break down the glucose to obtain all the individual carbons that are present. In terms of quantities, what does this amount to if one uses NMR instead?

Dr. Matwiyoff: We can do it with 0.2 μ M contained carbon-13. The sensitivity figures will probably change within the next year or so, driving the detection level down somewhat.

You mentioned the glucose. It is interesting because I think Chuck Gregg will discuss some ideas we have in looking at glucose metabolism. It does turn out that all of the six carbon atoms of glucose are readily distinguishable. By using carbon-14 you can degrade the molecule and distinguish each carbon-14 atom in the molecule. But what one can do in principle with NMR technique is something you cannot do with carbon-14. We can analyze for the situation in which two carbon atoms are adjacent.

You have a series of molecules in solution, isotopic isomers with carbon-1 label only, carbon-2 label only, and carbon-1 and carbon-2 labeled simultaneously. That is duck soup for us to analyze.

Dr. Sweeley: The number of differently labeled molecules made when you have only 90% carbon-13 would be rather high in a molecule like glucose with six carbon atoms. I don't know what happens in the mass spectra--I have the feeling that the number of lines one would see would be large.

Dr. Fales: In reference to the carbon-14 problem it is a little like beating a dead horse, because it never happens that we have two atoms of carbon-14 in the same molecule. In fact, a great deal of precaution is taken not to do this. So, one is always sure that there are no molecules truly doubly labeled with carbon-14.

Chairman Goldstein: Even with single labeling, and noting that the position of these peaks is relatively constant but allowing for solvent and concentration in sensitive cases, a great variety of applications can be made almost without any problem. You have then a list of numbers which you can follow through the metabolic process. Even with single labeling it is possible to get an answer. Double labeling, I think, is a luxury most people won't be able to afford for some time, but single labeling can carry you a long way.

Dr. Morgan: What you say is perfectly true. I would like to point out, however, the real advantage is one of being able to recognize the two carbons that are together after having been through a long metabolic pathway. This can be an extremely important feature.

The same thing is true for nitrogen-15 and other magnetic nuclei. I think it is important to emphasize here, not the things that are competitive, but the things that are unique to a particular technique. I appreciate the fact that people don't yet have carbon-13 to make doubly-labeled compounds, but I think double labeling is one of the important aspects of this technique.

A number of years ago I had an argument with a very eminent stable isotope chemist who could not see the advantage to making carbon-13 at the 90% level. He said you can do every thing at the 10% level and should concentrate on doing everything at that level. My point to him, at that time, was that there was a unique advantage in having both kinds of material.

Specifically, in connection with one of the slides that Matwiyoff showed a few minutes ago, there is a lot of normal acetate in that sample of blood plasma you showed that was not put in as label. The specific label characteristic of that was the twin carbon-13, which means that they occur at different resonance positions than the natural acetate which enables specification of the label.

Chairman Goldstein: I didn't mean to contest that advantage but simply to try to encourage people to look at the NMR techniques even if they were only able to label them at the 10% level, because there is a great variety of problems that can be attacked there at the outset.

The emphasis here on carbon-13 should not obscure the fact that some very good applications of nitrogen-15 have been carried out using Fourier transform techniques.

Dr. Borg: I am sure everybody in this room has pointed out the various backgrounds represented by the people here. On the other hand, there are many outside of this room who would like to get their hands on carbon-13 for NMR applications of which Matwiyoff has just given you a glimpse. Those people are ready to go. Clinical people are wondering where to use carbon-13, but the NMR people are champing at the bit to acquire some.

The sensitivity of NMR is very low, and some of you are asking questions about whether we can put carbon-13 in a biological organism and get out some component thereof and find out where the carbon-13 is. It might be well to emphasize again the relatively high concentrations and low sensitivity required for these purposes.

Chairman Goldstein: You can get more sensitivity if you are willing to spend more time and use more sophisticated techniques. In the future the superconducting system appearing on the horizon will give another factor of 10 in sensitivity. It is going to be costly but it won't be impossible if you want to work at it.

Dr. Matwiyoff: I agree that NMR is not a tracer technique, but I assure you that in any test load situation the technique will be valuable.

Chairman Goldstein: Much is made of the low sensitivity and, of course, it has to be admitted that in absolute terms it is low; but isn't it true that the time requirements and the instrumental requirements really reflect what the spectroscopist is doing? He is sweeping over a wide range trying to get every peak. Suppose he did some of the things talked about today. He would then select one peak and sweep over a much narrower range with corresponding improvement overall. Would you agree to that?

Dr. Matwiyoff: Yes, with this exception, one can narrow the spectroscopic window to search for a particular peak. If there happens to be another strong peak in that region, in the Fourier technique, we probably would get a foldback of the strong signal. We are now working on digital filtering techniques to try to solve that particular problem.

Dr. Hoffman: In the case of the antibiotic structure with carbon-13, do you recall the amount of the sample and the number of scans you used?

Chairman Goldstein: The amount of sample used was approximately 1 g. The number of scans was almost uncountable because it was done without pulse techniques. Again, it was swept over the entire region. Had we concentrated on one peak simply to identify it and had we used Fourier transform in addition, we could have gotten by with much less.

You must remember proton magnetic resonance (PMR) is far more sensitive. The figures I have recently heard on PMR with Fourier transform are, that in good cases, 10 μ g can be handled. The problem in the high sensitivity PMR region is clean-up, when you have low sample concentrations all the other materials present will interfere with the spectrum and you will have other problems when you get too sensitive.

Dr. Biemann: You don't need heavy isotopes to determine the structure of a compound. What surprises me in this discussion is that some of these techniques have been around for 10 or 15 years. For example, if we want to find out whether a carbon-nitrogen bond is broken in a molecule, we use carbon or nitrogen labeling and look at the mass spectrum and see whether you have a nitrogen-15 entity or not.

I think the main problem in all metabolism studies is that one has to fish the stuff out of a very dilute solution, which is not just a solution but is kind of a mess. It might be, in many cases, diluted by endogeneous material. Then one really battles sensitivity and detection limits which are major problems. Structural information is relatively easier to get.

Chairman Goldstein: I think Dr. Gregg may have some comments to add from his own experience that may be related to that.

SOME APPLICATIONS OF ^{13}C NMR TO
BIOLOGICAL PROBLEMS, PAST AND FUTURE

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My own principal interest is in the area of intermediary metabolism and I should like to express what I think are some of the features of NMR as they relate to the determination of metabolic pathways.

Figure 1 is from a 1970 paper by Tanabe, Hamasaki, and Seto of the Stanford Research Institute and Roy Johnson of Varian Associates (1). It shows the ^{13}C NMR spectrum of a compound known as sterigmastocystin, a precursor of the aflatoxins. The structure of the compound is shown in the upper right corner. The purpose of the NMR study was to investigate which carbons of acetic acid were the source of various carbons of this fused ring structure. This experiment could clearly be done with ^{14}C , the principal problem would be to develop the necessary carbon-by-carbon degradation of the molecule, a process which might reasonably be expected to take months.

The NMR experiment involves incubating one batch of the organism with acetic acid labeled in the carboxyl carbon with ^{13}C and a second batch with acetate labeled in the methyl carbon. The compound is isolated from both batches and the ^{13}C NMR spectrum run. The top spectrum in this figure is that of the compound following administration of carboxyl-labeled acetate, the bottom spectrum is that for methyl-labeled precursor. The peaks in both spectra are assigned by analogy with the spectra of compounds of similar structure.

Thus, examination of the upper spectrum shows which carbons in sterigmastocystin arise from the carboxy-carbon of acetate and the lower spectrum gives the same information for the carbons from the methyl group of acetate. The information gained is summarized in the structure in the upper right in which carbons originating from the carboxyl-carbon are shown as solid circles while those arising from the methyl carbon of acetate are shown as solid squares. While the same information is obtained with ^{13}C as would be obtained with ^{14}C , in one case the time scale is hours while in the other it is weeks or months. A

fringe benefit is that the ^{13}C method is non-destructive; spectra can be re-run *in toto*, or a particular region can be concentrated on if need be, while with the chemical degradation, if a particular step gives ambiguous results, it is difficult to back up and do it over on the same sample.

TABLE I
RANDOMIZATION OF LABEL FROM INFUSED GLUCOSE-2- ^{14}C
IN RAT KIDNEY GLYCOGEN AFTER TWO HOURS (2)

^1C	9.0
^2C	100
^3C	9.5
^4C	0.5
^5C	7.4
^6C	0.2

A similar situation is shown in the above table. Here glucose labeled with ^{14}C in the 2 position was infused into rats. After two hours, kidney glycogen was isolated, and degraded carbon by carbon to give the specific activities shown here. This is the work of Hostetler and Landau (2), done a few years ago; the objective was to assess the contribution of the pentose phosphate pathway to glucose metabolism in kidney. Here again this experiment could be done with glucose specifically labeled with ^{13}C in place of ^{14}C and the redistribution of label assessed by NMR. As in the first case, ^{13}C could be regarded as substitute for ^{14}C , yielding the same information at a lower expenditure of time, effort, and material. Actually the NMR method yields more information than does the ^{14}C method. When the carbon-by-carbon degradation is complete one gets the sort of information shown on this slide, for example that carbon-1 has 9% of the ^{14}C labeling of carbon-2. On the basis of such information one goes through a convoluted argument to derive the desired number, namely the percentage of glucose being metabolized via the pentose phosphate pathway. There is additional information available from the glucose labeling pattern which is not apparent from the ^{14}C degradation data but is revealed in the NMR spectrum of the ^{13}C compound.

For example, if carbons 1 and 3 of glucose have about 10% of the isotope content of carbon-2 does this mean that all glucose molecules have carbon-1 labeled to 10% of the level of carbon-2, or does it perhaps mean that 20% of the glucose molecules have carbon-1 labeled to 50% of the level of carbon-2? There is no way for the degradation method to distinguish between these possibilities while, because of the effect of neighboring ^{13}C atoms on each other, the NMR method can make such

a distinction. I don't wish to belabor the point; let me say only that data based on the carbon-by-carbon degradation contain less information than that based on ^{13}C labeling and NMR spectroscopy.

Let me now describe another experiment which, in principle, permits the simultaneous measurement of the rates of hemoglobin synthesis and degradation in an intact animal. Figure 2 shows the result of administering glycine labeled with both ^{13}C and ^{15}N to an animal and the resulting incorporation into the heme groups of hemoglobin. If one selects a peak in the NMR spectrum and measures the peak area in a series of sequential samples, a curve can be drawn whose slope is the rate of hemoglobin synthesis or, more precisely, heme synthesis. Figure 3 shows the pathway of heme degradation, the tetrapyrrole ring is opened enzymatically and a mole of carbon is excised. This carbon appears as carbon monoxide and is the source of most of the carbon monoxide in the body. Figure 4 shows the fate of the carbon monoxide which is bound to the heme iron of hemoglobin and because of its chemical environment gives a unique peak in the NMR spectrum. Measurement of this peak area as a function of time gives a curve whose slope is the rate of degradation of hemoglobin. With a little experimental manipulation, the NMR spectrum need only be run on a single sample for each set of time points and the results on a series of samples then give both the rates of synthesis and degradation.

This is a Gedanken experiment at the moment, although we hope soon to begin preliminary investigations. I cite this approach primarily to show the potential utility of the NMR technique in acquiring kinetic data on complex living systems.

To summarize: I think that NMR spectroscopy of the ICONS elements can potentially provide to the biochemist, clinician, and pharmacologist information equal to, and in some cases much better than that available from the corresponding radioactive tracers where they exist. Furthermore, suitable radioactive tracers do not exist for oxygen and nitrogen. Moreover, this information can be obtained without destroying the sample and very much more quickly than by the chemical degradation methods. I don't think it requires either unusual courage or exceptional vision to predict a brilliant future for this technique in the life sciences.

REFERENCES

1. Tanabe, M., Hamasaki, T., Seto, H., and Johnson, L. Biosynthetic Studies with Carbon-13: ^{13}C Nuclear Magnetic Resonance Spectra of the Metabolite Sterigmastocystin. *J. Chem. Soc. (C)* 22, 1539-1540 (1970).
2. Hostetler, K. Y. and Landau, B. R. Estimation of the Pentose Cycle Contribution to Glucose Metabolism in Tissue *In Vivo*. *Biochemistry* 6, 2961-2964 (1967).

Figure 1. ^{13}C NMR spectrum of sterigmatocystin in dioxan: (A) from $\text{CH}_3^{13}\text{CO}_2\text{Na}$, 30 mg/1.0 ml with 63 scans of 5030 Hz at 200 s/scan; (B) from $^{13}\text{CH}_3\text{CO}_2\text{Na}$, 27 mg/1.0 ml (saturated solution) with 1055 scans of 5030 Hz at 200 s/scan. The labeling pattern in the structure of sterigmatocystin indicates the precursors as C^{13} from $[1-^{13}\text{C}]\text{acetate}$ and C^{13} from $[2-^{13}\text{C}]\text{acetate}$. * Indicates C-C coupling between C-9 and C-15.

Figure 2. The incorporation of glycine doubly labeled with ^{13}C and ^{15}N into the porphyrin ring of hemoglobin in an intact animal.

Figure 1. The pathway of heme degradation. One of the methine carbons of the porphyrin ring appears as carbon monoxide.

Figure 4. The binding of carbon monoxide to the heme iron of hemoglobin.

DISCUSSION

Dr. Fales: This sort of thing comes up quite often. What is the best possible experiment to run with carbon-13? If I were looking around for a really great experiment to run with carbon-13 acetate or whatever I could buy, I would get hold of an antibiotic study and I would put the sodium acetate into a culture which I had worked out to effect complete conversion, or as near complete conversion as I could, and then I would fish out from the broth a few milligrams of the antibiotic and look at it by NMR. That is a beautiful study. Similar ones are coming out in the literature these days, and they are really elegant because they represent a very rapid way to get into the biosynthesis of antibiotics.

I do think we have to distinguish between that kind of experiment and the experiments that I think a fair number of people here are involved in, which is in the first case drug metabolism and in the second case intermediary metabolism, where recoveries are so much less. That is to say, in antibiotic conversions, I think probably you could put in 27 mg of sodium acetate and hope to get out a few mg of antibiotic, maybe; but if you feed 27 mg of carbon-13 labeled phenylalanine to a human being, recovery and identification is difficult.

On the other hand, perhaps if we feed 27 mg of carbon-13 labeled drug, and fish out the undiluted material, as pointed out earlier, we still have the possibility of studying the product. The only problem is that we are not going to get out anywhere near 1 mg of product.

When you have 1 μ g it is almost an axiom that by the time the pharmacologist has a pure crystalline metabolite isolated, its structure was already known some months back. They know the structure by the time we have the crystal, by virtue of gas chromatography-mass spectrometry.

The things that happen to drugs metabolically are relatively simple. The structural problem there is not the way it is in the antibiotic situation. I would like to hear more comment on that.

Chairman Goldstein: Suppose we invite the panel each to comment from his own vantage point.

Dr. Gregg: I agree. I think there are many cases where experiments can be done essentially in a loading situation, where you are talking about milligram quantities. When you are talking about micrograms in drug metabolism, then you are stretching things to the limit. There are ways around that. There are several things you gain by NMR technique. It may very well be that in some cases you may not have to go to the purity normally required in order to characterize it.

Another aspect is that you can trade off time. If you put it in at 5:00 PM Friday and have your information Monday morning, the fact that it has taken 48 hr+ is not really too devastating, especially considering the alternative.

Dr. Fales: In my experience at NIH, when a sample is brought to me from an honest-to-God living experiment, either with a rat or a human being, there is always a little bit of brown material in the bottom of the tube. (Laughter) It is then no longer a structural problem--it is usually a quantitation problem.

We have invested heavily in carbon-13 instrumentation too, and the Fourier transform is going to save everybody's life. However, Fourier transform is not the end/all of this problem. Carbon-13 labeling certainly helps us because it gives us another factor of 100 in sensitivity.

Chairman Goldstein: What size sample do you use?

Dr. Fales: Fifteen mg, but one would never foresee using those larger sample sizes in biological experiments. Probably we will have to go back to smaller sample sizes. We are also very enthusiastic about it, but I do think, in view of the money that it costs the taxpayers of the United States (at least our instrumentation did), one should try to think of alternative methods as well.

Chairman Goldstein: I don't think there is any basic disagreement here. It would certainly seem that the methods being discussed are complementary rather than antagonistic. The ideal laboratory would be one that had an excellent mass spectrometer in one room, and the very finest NMR in another room. You can't rule one thing out because another comes on the horizon. I hope there would always be some room for new techniques to arrive and take their place. This is not to say that the cost of the effort in the NMR technique is not justified, and because it can't handle all the dirty problems doesn't mean it can't justify itself.

I support very strongly, the speaker and the panelists' remarks that NMR has its own advantages. These advantages are not, in general, replaceable by other techniques because, as Matwiyoff has adequately illustrated, the fine-grained character of NMR isn't reproduced by anything else. Let me give you an example. You can watch conformations change with pH and with temperature. It is an irreplaceable technique where it is applicable, and it provides very, very sensitive measures of the details of molecular structure.

Another example is binding sites: Certainly we can study binding sites as with any other technique--or exchange phenomena.

Dr. Morgan: I would just like to re-emphasize what Dr. Borg and you have said. This is largely in reference to a question Dr. Biemann answered earlier. At several conferences I have been to recently, the

question has seriously been raised with regard to all these biological materials, studied by x-ray techniques and electron diffraction, as to whether this represents the real world or not. When you isolate a crystal of something, it is distinctly separated from its environment. What you want is the conformation and the structure of this material in its actual environment.

You would like to know not only the structure but also the dynamics. I don't think we are there yet on some of the very complex things, but it is in sight. One of the things that is frequently overlooked is not just the gain in sensitivity but in dimension because you not only get information about the structure of the material but you get the time-dependent behavior at the same time. This is exceedingly important and is a very real problem.

Many of you were at the conference on the West Coast several years ago when the whole question of direction came up with regard to increase in sensitivity. I think the answer there, reinforced since, is that you don't go in any one direction--you go in all directions. You go from a single-scan spectrum to signal averaging. You go from signal averaging to Fourier transform. Then you go to high abundance materials.

One thing not mentioned here is the very real possibility of synthesizing the entire protein with depleted carbon-12 so that it has no carbon-13 in it at all or a very low abundance of carbon-13, and then introducing your specific label. The sensitivity is then greatly enhanced.

Chairman Goldstein: I am glad you brought that point up.

Dr. Fales: One last word. Before I am completely misunderstood, I am grateful for carbon-13. I think it has a great future for its own sake because it allows us to do the very thing you gentlemen have been talking about. But I also know for a fact that there is a great feeling on the part of the clinical pharmacologists that what they really want carbon-13 for is not for the things we have discussed here but replacement for carbon-14 as a tracer because of the radiation hazard and all of my remarks are only meant to reflect on that one point.

By itself, for things that carbon-14 won't do, I am 100% with you; but I worry about the use of carbon-13 as replacement for carbon-14. It seems to me we don't have to really say it is a replacement for carbon-14 to praise carbon-13, to do something constructive with it, and I don't want to get in the position of trying to be forced to say carbon-13 is ever going to replace carbon-14.

Chairman Goldstein: One of the advantages of the NMR technique in general is that it has greater flexibility than any other spectroscopic technique I have ever heard or read about or have known about. There are more different kinds of things you can do with it and you can tackle nucleus after nucleus.

Instead of looking at that particular problem in terms of carbon-13, alone, it may be helpful to turn back to protons. The proton is a stable isotope and it will pick up a lot of sensitivity especially if somebody happens to have the newer superconducting systems around, you will be surprised what can be done.

Dr. Borg: I agree with everything that has been said. I want to add just one word. Dr. Fales' perspective is one I am glad to hear articulated explicitly. For those who are life scientists here, I don't want to confuse the spectroscopic picture, but there is another spectroscopy which is champing at the bit to use carbon-13 substitutions for other kinds of structural work, and this is ESR or EPR. This is limited to paramagnetic materials; but as many of you know, there are many such either in existence or that can be made to exist in biologically interesting molecules, and the same kinds of considerations that were brought to your attention with regard to NMR apply. Carbon-13 as a replaceable nucleus is of great value.

Paramagnetic materials may be rare, but the sensitivity of EPR is about three orders of magnitude higher than NMR, so that for those questions where EPR is a good technique one can work with much smaller amounts of material.

Chairman Goldstein: Dr. Gregg has a few more comments that he would like to get out of his system.

Dr. Gregg: I would like to correct an oversight. Dr. Robinson pointed out this morning that there are other isotopes in the program, and these are oxygen, hydrogen, and sulfur. Most of us at Los Alamos tend to talk about carbon-13 as sort of a code word which to us means all of these things. I would like you people to remember that there also are NMR techniques applicable to nitrogen-15 and oxygen-17. There are no radioactive isotopes for either of these elements, so this is something else that I think is worth keeping in mind.

Dr. Morgan: Dr. Borg gave half of my concluding remarks. As an EPR spectroscopist, I usually end up the discussion by saying, "Next time let's talk about EPR." I do think a number of things have been said and implied about various applications of nuclear magnetic resonance to the use of carbon-13, but I would like to bring some of these together with a plea to those of you who are clinically oriented.

It seems to me, in listening to the discussions, that a high percentage of the techniques that are now in use have features which are dictated by the nature of the technique; that is, there is a big backlog of applications which are oriented in such a way that they make the best use of carbon-14, and that is not necessarily the best use of carbon-13. There is a heavy emphasis on the isolated small molecule, because detection techniques are best for those. Mass spectroscopy is oriented heavily in the direction (at least for high sensitivity detection) of the CO₂ molecule or corresponding small one-carbon molecules.

If we turn our thinking around with respect to clinical applications, and ask specifically what kind of a thing would you attempt as far as analytical methods are concerned, given the specific attributes of nuclear magnetic resonance applied to this problem, where would we end up? I think some creative thinking has to be done in that direction, instead of trying to apply techniques that are best applied in another way.

WEDNESDAY EVENING SESSION

Chairman: B. N. Tolbert

WEDNESDAY EVENING SESSION

Chairman Tolbert: The primary purpose of this evening's meeting will be a synthesis of ideas and information. Later, I hope we can consider possible applications of carbon-13. We have various people who are experts in given subjects--for instance, Dr. Matwiyoff, who is an expert in carbon-13 NMR--and they can comment on how problems might be approached or discuss whether carbon-13 would be appropriate for their solution.

I prepared a few notes which I will briefly review.

GENERAL DISCUSSION REGARDING STABLE ISOTOPES

By way of a short historical review, I might point out that the AEC has always been interested in stable isotopes. For example, there is the Calutron at ORNL and the isolation and sale program at Mound Laboratories. The pace of this program, especially in regard to carbon-13, was accelerated in 1968 when the Division of Biology and Medicine undertook, through the Los Alamos Scientific Laboratory, to develop this and other isotopes for scientific use. The development was based on an on-going program of stable isotope separation and application in Dr. E. S. Robinson's division. The initial objectives of the program were to produce enough carbon-13 to study toxicity in several biological systems and we will return to this subject later. Other objectives include development of instrumentation and synthesis procedures and an evaluation of all scientific aspects of this problem. In more recent times the program has been expanded to include the stable isotopes of N, O, and S.

Part of the output of the carbon-13 enrichment plant is currently being sent to the Mound Lab for sale. This is the source of a major fraction of the carbon-13 now being used for starting material by companies specializing in synthesis of labeled compounds. If you are not on their mailing list and desire to be, you should write to Mound Laboratories, Miamisburg, Ohio. There is considerable interest by commercial firms in this development and I think the availability of labeled substrates will improve greatly in the next few years.

The purpose of this seminar is to consider the ways that the stable isotopes--carbon-13, nitrogen-15, oxygen-17, or oxygen-18 in particular--can be used in the biological sciences with emphasis on pharmaceutical and medical applications. Specific technical aspects

of this seminar have include: isotope analysis, enrichment and availability, synthesis of labeled substrate, instrumentation potential, and development needs as they relate to present and foreseeable problems. Specific information on many of these subjects has already been covered in the earlier presentations.

1. *Availability of isotopes* For carbon-13--fair now, getting better--availability should be excellent in the future for all isotopes except oxygen-17. Nitrogen-15 and oxygen-18 separation technology is now known and nitrogen-15 and oxygen-18 should be readily available in 5 years. We will presume enriched raw carbon-13 and nitrogen-15 costs will fall to the deuterium price range. There should be a good inverse relationship between cost of the isotope and the scale of preparation for a long time. Thus prices should decrease if widespread clinical use develops.

2. *Licensing of medical use of the isotopes* At present there is no reason to believe that the carbon, nitrogen, or oxygen stable isotopes are toxic in any concentration. It seems probable that the toxicity of deuterium is an unusual phenomenon related to its bonding role in macromolecular and membrane conformation. Therefore, Food and Drug Administration approval of labeled compounds on an experimental basis should be readily available, and it should be possible to generate satisfactory information which would be the basis of authorization of routine clinical use of stable isotopes. However, generation of zero toxicity data is a difficult job today and conversations with Food and Drug must be initiated before serious studies are undertaken. Furthermore, someone must finance such studies. I am tacitly assuming that licensing will be required, but this point might be contested.

3. *Synthesis of labeled compounds* Chemical and biological synthetic procedures for preparation of most labeled drugs are known, although much technological development is needed. Some new innovations are necessary, but this aspect of stable isotope use is not a limiting one. The financial incentive of a good market for compounds labeled with stable isotopes will hasten their production.

It is important to note that synthesis of labeled compounds will not be done on a radiochemical scale, which is commonly on the millimole scale. Rather, such syntheses will be made first on a 10^2 to 10^3 g scale and later on pilot plant scale of 10 to 100 kg per batch. This means that the technology is not to that of radioisotopes, although the marketing effort will be directly comparable.

One gram of carbon-13 incorporated into a compound of M W. 130 gives 10 g of product--maybe enough for several patient experiments. Multiply this 10 g by any reasonable number of patients and investigators, and one is soon in the tens of kilos range--or that of the fine chemical plant. For instance, in 1966, according to a survey made for

the National Center for Radiological Health, 4,500 patients were "treated" with carbon-14 and 7,500 with tritium to make a total of 12,000 patient experiments. Since there are no toxicity handling problems in working with stable isotopes, it seems possible many firms might get involved in synthesis. The distribution as pharmaceuticals will surely develop through existing pharmaceutical houses.

4. *Analysis of stable isotopes* Analytical procedures for carbon-13 and nitrogen-15 are not only the greatest problem area in using these isotopes, but are also full of promise and exciting prospects. On the negative side, all analytical procedures for stable isotopes, except infrared, presently involve expensive equipment, and sophisticated operators and are suitable only for a small number of samples. On the positive side, most analytical procedures give much more information than is obtained using radioactive isotopes.

Thus carbon-13 NMR gives position of label as well as enrichment. Mass spectrometer measurements also give information on distribution of isotopes within a molecule, as does infrared.

I have great faith in instrument developers and manufacturers. I remember only too well the crude G. M. tube under which I slid my first carbon-14 samples. Compare this to the present 200-sample automatic liquid-scintillation spectrometer that counts three isotopes at one time! If we have a good medical or scientific reason for building special-purpose analyzers, they can and will be constructed.

The specific problems in the area of stable isotopes are: (a) instrument complexity (b) instrument sensitivity (c) sample handling capacity.

The principle instruments that we can presently consider include:

- A. Proton NMR--in which carbon-13 can be detected only if a proton is attached to the carbon-13.
- B. Carbon-13 NMR--a powerful method, very new to the chemical and biological world, but still lacks sensitivity for biological samples.
- C. Infrared analysis--a method which can give rather excellent results in simple molecules, and may be improved.
- D. Raman spectra--which has yet to be applied to carbon-13 assays, but should be more sensitive, specific, and easier to use than infrared (mostly because one can work in aqueous solutions and use laser sources).
- E. Mass spectrometry--a very sophisticated instrument that could be and has been simplified for specific use, such as carbon-13/carbon-12 ratio in breath CO_2 . This method is by far the most sensitive of the carbon-13 assay techniques but it is also one of the most complex, both to operate and interpret.

- F. Activation procedures--this rarely-discussed method has been studied by one group at Oak Ridge National Laboratory and it can readily be shown that selective activation of carbon-13 in the presence of carbon-12 occurs when the bombarding particles reach the medium energy range of nuclear physics 2 to 20 meV. This is a gross procedure, but very small samples can be studied. It is also a bulk method that does not require a chemical knowledge of the labeled substrate to give results. It probably is not competitive with burning a sample to CO₂ followed by a mass spectrometric assay on the product.
- G. Density measurements--in DNA or high molecular weight proteins a 3% increase in carbon-13 or oxygen-18 gives sufficient change in buoyant density that such molecules may be separated using a density gradient centrifugation process. The molecular biologists have already used this to define newly-synthesized DNA, and the method has intriguing aspects for human studies. Rather substantial amounts of labeled precursors would be required in human studies.

I personally think it's a bit presumptuous for me to discuss the most likely aspects of medicine and clinical pharmacology in which stable isotopes can play a part. Nevertheless, to stimulate discussion in this area, I would like to outline some of these applications.

Analysis of clinical abnormalities by measurement of respired ¹⁴CO₂ from labeled metabolites has been carried out a number of years. Some of the best work has been done by Winchell and Pollycove in California, by Baker and Shreeve in Brookhaven, by W. Gordon in Wisconsin, by G. V. LeRoy at the University of Chicago, and by Schwabe and Bennett at UCLA. All of these workers showed that one could do useful clinical assays by this method but the procedure was never adopted on a routine clinical level. Why not? First, essential information could usually be obtained by other methods; second, the equipment was fairly complex. Most of all, I think there was fundamental opposition to the use of radioactive isotopes in nearly-normal individuals. The obvious safety of carbon-13 augurs well for its use in such applications.

Pool size determinations of metabolites are of importance in a number of clinical studies. As an example, I am interested in the pool size of ascorbic acid in normal adults on various dietary regimes. The pool size of ascorbic acid is about 1,500 mg and after ingestion of labeled ascorbate, the pool reaches equilibrium in a few hours. The urinary ascorbate can be assayed for ascorbate-carbon-13 and the pool size determined by isotope dilution. We are presently working on this problem and hope to apply the procedure to other vitamins. We cannot do such studies using carbon-14.

I would suggest that the pool size and turnover of a number of the tryptophan and tyrosine metabolites that are important in neurological abnormalities could be determined using carbon-13 to provide clinical tests for some mental health problems.

The rate of synthesis of macromolecules is an aspect of human metabolism that is almost unexplored by clinical procedures, although many tests are made of enzyme and protein levels. It seems reasonable to assume that, by use of a stable isotopically-labeled essential amino acid, this rate of synthesis and turnover could be measured for readily available tissue,--e.g., blood.

To summarize: the availability of carbon-13 at the present time is good. The output of the Los Alamos plant, as Dr. Robinson mentioned, is running approximately 7 kg a year. It should increase in the future. There is some interest by foreign countries in this topic and the English have a reasonable-size plant going into operation under the British Oxygen Corporation. The Russians have been making carbon-13 for some time, but I know very little about their total output or their enrichment levels. Nitrogen-15 and oxygen-18 are not difficult to enrich, but at present they are not available in quantity.

The price at the present time is much higher than it could be if one considers the economics of the separation based on basic thermodynamic data. The energy of separation is small and a large-scale system should eventually push the price of carbon-13 down toward the present price of deuterium. Remember that deuterium is a rarer isotope and yet we buy it for a few cents a gram. If carbon-13 were used in very large quantities, such as one might anticipate with a medical application, there is no reason that the production cost per gram should not fall to less than \$1 a gram. This is a very important figure to remember.

Finally, in regard to the question of licensing of stable isotopes for medical use, I personally believe there is no reason to suspect toxicity. This morning we passed rather rapidly over the mouse feeding experiments that had been done at Los Alamos on the toxicity of carbon-13. I want us to take another look at the data which I think are important and Dr. Gregg, who has been conducting those experiments together with other members of the Laboratory, said he would review them.

Dr. Gregg: A number of things have been done in the course of the carbon-13 program at Los Alamos. The first experiments involve the raising of algae, green algae, on 90% carbon-13. The result of this experiment, which was similar to the result reported by the group at Argonne, showed no decrease in the growth rate of green algae on 90% carbon-13, and no discernible morphological changes.

In the mouse feeding experiment the food was yeast. We raised about 1.5 kg of yeast on 90+% carbon-13. There again there was no increase in the generation time of the yeast and no discernible abnormality of growth.

At an early stage it was decided that we would also have to investigate mammalian systems. Initially it was proposed that we would start with a small number of mice, two to be exact, and work our way

up to a larger number as our production facilities and our capability of doing the experiments progressed. Two mice would appear to be a pretty trivial operation, but in point of fact, it is not. A mouse requires a minimum of about 3 g of yeast per day, so this is about 100 g of carbon-13 yeast per mouse, per month. To grow this amount of yeast requires about 3 times its weight in carbon-13 labeled acetate; so if one feeds two mice for one month, that is 200 g, of carbon-13 yeast and this requires 200 g of yeast, which in turn requires about 600 g of 90% doubly-labeled carbon-13 acetate.

The yeast has to be cultivated, and when we began there was considerable pressure on the biomedical research group to get this experiment started; we started long before we were really ready to do so by raising the yeast in a couple of 14-liter fermenters. Subsequently we went to a 200-liter fermenter which made things much simpler.

Maintaining two mice consumed a fair fraction of the time of a number of people; something of the order of four or five Ph.D.-level individuals were involved simply to keep these mice fed.

It became increasingly apparent that raising mice on a high level of carbon-13 was probably not going to convince anybody of anything. What probably made more sense was to wait for the selection of the clinical application, and then to concentrate our efforts in toxicity on the particular compounds in the particular dosage proposed.

Therefore, this mouse experiment was terminated--part intentionally and part by an act of God. In the course of taking a breath sample on one of these mice, it died of unknown causes.

Chairman Tolbert: I thought it was asphyxiation.

Dr. Gregg: It was not. The mouse was autopsied, samples were taken for detailed examination and according to our veterinary pathologists there was no discernible cause of death.

Chairman Tolbert: Can we have the initial and final weights of the mice?

Dr. Gregg: About 14 or 15 g initially. They were 6 weeks old. They were taken shortly after weaning. They were put on a diet of 90% carbon-13 yeast with no preliminaries. We went right from natural abundance to the highly-enriched level. Their weights rose to a plateau level of about 27 or 28 g. At this point we were feeding the mice 3 g of yeast a day. That turns out not to be enough to get optimal weight gain. It should have been closer to 5 or 6 g.

Dr. Hurwitz: Did you feed identical mice with a yeast diet which was not carbon-13?

Chairman Tolbert: Were they pair-fed?

Dr. Gregg: No, these mice were fed yeast *ad lib*. The first mouse was fed from the first of February to approximately the end of May, and the second mouse was fed through the end of August. So, it turns out to be about one mouse-year (Laughter). On the basis of breath analysis, this gives a minimum number for the carbon-13 concentration in these animals; about 73% of the body carbon was carbon-13. Two mice are not an imposing set of statistics, but that was all we could do in the circumstances under which we were operating and we elected not to go to a large number. However, I think it is still significant that two mice were raised from teenage, if that is the term, to adulthood. Their gross weight doubled on a diet of 90% carbon-13. There were no discernible abnormalities, and the final carbon-13 level in the body was not less than 70%.

Dr. Hurwitz: Did they have any litters?

Dr. Gregg: Yes. The second mouse was bred to a male outside the carbon-13 colony. She had two litters, each born over weekends when she wasn't being watched. In the first litter every baby mouse was eaten. In the second litter we found pieces of a couple of them. This is not unknown in mice, as you know; it may be that we would have had cannibalism in the mice on normal yeast too.

One of the problems in the early part of this experiment was that we had the mice in metabolism cages because we wanted to collect urine and feces. They were on wire and had to stand on their hind legs to get food.

Dr. Freese: Two litters is a small number for a mouse. How long did it take to get this mouse pregnant? Was there any delay?

Dr. Gregg: I think each time the male was in the cage just at night for a week.

Dr. Fales: If I were a lawyer for a group that was attacking carbon-13, I would say that this demonstrates that carbon-13 causes a 50% mortality in mice and that it kills by an insidious disease not heretofore recorded in the annals of medical science.

Chairman Tolbert: I think the answer has to be that we are presenting these data to show you that this is a very difficult experiment. Remember that yeast is not an adequate diet, but has to be supplemented with several of the amino acids. It is short on vitamins. It is impossible to do a statistically-significant experiment with two animals, but nonetheless I felt we needed to present these data because they are all we have.

Dr. Chidsey: Do you have histological sections on these mice?

Dr. Gregg: Yes. The pathologist took tissue samples for imbedding and we have the rest of the mouse in liquid nitrogen. We intend to do carbon-13/carbon-12 ratios on individual tissue to get some idea of the turnover rate.

Chairman Tolbert: There were no gross morphological differences?

Dr. Gregg: According to the veterinary pathologist, there was nothing unusual.

Dr. DeGrazia: Were any other microscopic procedures done?

Dr. Gregg: The answer is no, but we have the tissues.

Chairman Tolbert: The nature of the questions raised by the presentation of these data indicates that the toxicity of carbon-13 is a very difficult subject. The toxicity of nitrogen-15, oxygen-18, and perhaps even oxygen-17, must also be evaluated.

One of the purposes of the discussion this evening is to generate information that can be used in planning future work. I would like to ask Dr. Clark if he would discuss the problem of toxicity and licensing.

Dr. Clark: I will be as brief as possible. Essentially you asked for an hour's discussion in 5 minutes.

As you head into uses of chemicals in man, you have the necessity of satisfying the regulations of the Food and Drug Administration. Such regulations are not required for *in vitro* experiments or any animal experiments. My inferences here are only with respect to the administration of a chemical to a human.

In 1962 the Food and Drug amendments to the Food, Drug, and Cosmetic Act were passed with subsequent regulations. It should be understood that this body of regulations was designed as a safeguard in the development of new drugs. Specifically it requires filing an Investigation of New Drug application (IND) before its experimental use in humans can be undertaken. Secondly, before a new drug can be accepted for market, it must be proven safe and effective.

The procedure and requirements are clearly stated, and I will skip those, but for a *de novo* drug, a new chemical, this ordinarily means toxicity studies in perhaps several hundred mice, three different species of mice, rats, dogs and, if it is a contraceptive, ten years in monkeys. Don't let me frighten you. I am trying to give you just a little background. This also involves chemical identification and purity of the compound. It involves Phase I, II, and III clinical investigation. The reason for pointing this out is that the whole legal regulatory machinery is set up for this purpose. It was not designed to cover experimental tools or investigational chemicals that were not intended to be drugs.

There has been considerable discussion of what, short of collecting this great magnitude of data, could be done in terms of the clinical pharmacologist's interests: first, analytical tools and, second, diagnostic agents which are another class of compounds.

I cannot be definitive, but I can give you further ideas. It seems to me we have to approach this in terms of classes of stable isotopes and I am limiting my comments to carbon-13. It seems the safety requirements should be the least for naturally-occurring metabolic products such as citric acid and glucose. Two sugars have already been approved for diagnostic purposes with relatively little data.

Some preliminary discussions have been carried out with the FDA in this class of the work and their first thoughts are that chemical identification, structure, and purity are the minimum information required. What else will be required, I cannot really say.

The second class of these kinds of things would be marketed drugs, as a specific example: diphenylhydantoin. In this case there are preliminary assumptions that the chemical work is required showing that the compound is chemically identical (except for the isotope) with the marketed compound. What else is required, we don't know. However, in these cases it is my belief that any experiments to be done in man will require the filing of an investigational new drug application, which now requires a 30-day waiting period before you can proceed with the human experiment.

A third class of compounds are the metabolites of marketed drugs. We have not gotten a clear picture on this. However, this much is clear; not only must we have all the chemistry, the manufacturing details, and so on, but also some limited toxicity information. For example, three months of chronic toxicity in a group of rats, or perhaps three weeks for mice would, cover, say, single administrations, or three months for mice to cover one week's administration.

The fourth class, it seems to me, are new chemicals with intended therapeutic use, in which case you are committed to a \$10 million study.

That is a very bare outline. I will be glad to go further with respect to comments on it.

Dr. Weiger: Would giving carbon-13 glucose to man fall into the category of drug or diagnostic agent?

Dr. Clark: I think this falls in the first category of which I was speaking. It seems to me that carbon-13 glucose would require an IND application, but this would need to contain only the process of manufacture or the process of synthesis, purification, the criteria for identity and purity. I think this is the minimum that would be required. But a technical loophole exists; if the compound after manufacturing is not shipped in interstate commerce, it can be used within the state without filing an IND. I don't recommend this procedure, at least with respect to drugs.

Dr. Borg: Do you foresee that each separate substance which is labeled with carbon-13 will require a separate clearance?

Dr. Clark: If a class approach cannot be worked out, this means each individual compound will have to be cleared for that purpose.

In addition, let me reiterate, the IND contains the synthesis, the criteria for chemical identification, and a clinical plan. In other words, what are you going to use it for; how you are going to use it, in what dosages, and for what periods of time the compound will be administered. This will work toward some kind of class clearance of compounds; and I think we have made a little progress, because as you go down this list of classes as I have outlined them here, the more difficult it is going to be, I am sure.

Dr. Chidsey: I don't believe the FDA discriminates between radioactive compounds and non-radioactive compounds.

Dr. Clark: It is my understanding that, in the past, the clearance for investigational use of radioactive compounds has been in the hands of the Atomic Energy Commission and the committees it has set up. It is also my understanding that the Atomic Energy Commission has taken no action with respect to stable isotopes.

Dr. Totter: I think you are probably not correct; the law does give the Food and Drug Administration jurisdiction over radioactive compounds. FDA delegated this back to the Atomic Energy Commission, and that is why our committees have had some charge on it. About once a year they threaten to take back that delegation. I don't know exactly what the state of it is now, but they do discriminate between stable and radioactive labeling.

Dr. Chidsey: It is a very complicated situation, I realize. In one particular instance in our own laboratory we have administered a radiolabeled new drug, and the only difference this made to the Food and Drug Administration was that this compound be demonstrated to be chemically pure and identical to the drug we were giving these patients.

Dr. Clark: I am sure there are liberties taken with the literal interpretation. When you reach the stage beyond Saturday afternoon experiments in your own laboratory on one of your medical students and start shipping this stuff to somebody else, then you definitely come under the legal responsibilities.

Dr. Hurwitz: Who currently regulates diagnostic things like tritium and iodine-131?

Dr. Levine: The answer for the present is that the AEC is operating with an exemption from the FDA. The investigators using radio-isotopically labeled agents do not have to go through the IND new drug application route. FDA keeps saying that they will take over this procedure, but as of this year they still have allowed the committee on the human application or human use to do it.

It would seem to me that if there is a potential for carbon-13, the AEC might try to seek a comparable exemption at least for the foreseeable future until the FDA, which has many other fish to fry, can get around to taking over the radioactive isotopes.

Chairman Tolbert: There is one problem; the AEC has no exclusive prerogative in the production of stable isotopes, and in fact foreign production is already coming into the United States.

Dr. Totter: I think the reason the FDA has redelegated the authority to us is that they do not have the radioactivity experts on their staff that they need. They might consider themselves experts on stable isotopes; I don't know.

Dr. Clark: The conclusion to this is that it is not settled at the moment. Based upon this seminar, it would appear to be time for certain of us to meet together and to face the problem in terms of the stable-isotope drugs going into man. There are three classes of chemicals from a legal standpoint of chemicals. The so-called GRAS list is Generally Regarded As Safe. Saccharine fell into that list until it fell through the hole in the floor. There are many other food additives which fall into that category, but these are beginning to be looked at again. There was a class of old drugs which were exempt under grandfather clauses. These were mainly the drugs approved prior to 1938. However, as most of you know, drugs that were approved subsequent to 1938 were approved only for safety, and have recently been re-examined for both safety and effectiveness. Then there is a category of new drugs which can obviously be a brand-new chemical, or if it is an old drug with a new use it then becomes a new drug and is subject to IND. I think we have enough information from this meeting to try to zero in on some specifics.

Chairman Tolbert: May I summarize an earlier part of your commentary. I understand that if one desires to do experimental investigations in man, using stable isotopes, one should file an application with the FDA. The probability is that you will get no reply in 30 days, and then you are free to go ahead and do the experiments?

Dr. Clark: Broadly speaking, that is true.

Dr. Mirkin: I presume, Dr. Clark, that does not mean to imply that one bypasses regional or local jurisdiction of the committee at that institution supervising such administration. I think that is quite important, because such committees exercise a very significant restraining role. I am talking about the Committee of Human Volunteers or the Clinical Research Committee of any institution in which the investigation is to be carried out.

Dr. Clark: I am very glad you pointed that out. It should also be cleared by that institution's isotope committee unless that is part of the central committee. I might add that from the standpoint of support of any of this work by NIH funds, this is also important.

Dr. Tarlov: I would like to continue on the toxicity line. I wonder if some studies have been done in isolated enzyme systems, for example, to see whether or not the isolated enzyme will handle the

carbon-13 substrate in an identical way that it will handle a carbon-12 substrate. It seems to me this would be perhaps a less expensive experiment to do before the animal studies.

Dr. Klein: There are a number of experiments but they are usually with deuterium. You can detect a difference in the rate constant for decarboxylation reactions with carbon-13 with very careful measurements. Differences are the order of 1 or 2% difference. It is not a large effect.

Dr. Clark: They are very small, and they are usually best demonstrated in a closed system, which obviously a biological system is not. If you let an enzyme run to exhaustion on the mixed substrate it will preferentially use the carbon-12 and it will leave the carbon-13 behind in a very small degree of enrichment. In a biological system you have throughput of the whole thing and you don't exhaust your substrate; it is flowing through. You have an open system and consequently the enrichment effects are really quite small.

Dr. Klein: It is not a matter of accumulation. The carbon-13 would lag a little bit. That does not mean it builds up.

Dr. Mirkin: This could be a cascade type of effect.

Moving away several large steps up into an integrated organ system, the effect of a deuterated compound (and I am speaking of epinephrine) is quite different from the normal norepinephrine effect in terms of contracting smooth muscle. This is an observation of some years ago. I have never seen this followed up, and I wonder if anybody at this seminar is familiar with it. What they found was that the biological effect of a deuterated compound was quite different from the normal compound. This is with respect to norepinephrine.

Dr. Klein: Deuteration was very close to an active site, and I think it was a group of Belgian workers who studied the phosphorylation of tritiated thymidine and found a considerable difference. But it was because cleavage of an isotopically substituted bond was involved, and this was in a closed enzyme system. This is not the same thing *in vivo*.

Dr. Jenden: We have made an exhaustive examination of isotope effects in three isotopic variants of acetylcholine, one of which was deuterated in all of the methyl groups surrounding the nitrogen, and there were no detectable differences between these three in regard to V_{max} , cholinesterase, or nicotinic activity.

Chairman Tolbert: The effects with the deuterium labeled compounds are entirely different than with carbon-13 or nitrogen-15, first, because of the mass of the material and, second, because even a $-CH_2-$ is involved to a certain extent in hydrogen bonding within the molecules. This changes the picture completely. A 90% carbon-13 enzyme like acetyl cholinesterase has not yet been studied.

Dr. Totter: In the last couple of years we have been strongly challenged on the possibility of isotope effects of tritium, and we went to the literature and looked for appropriate studies on deuterium. Few of them are applicable to this problem, because analysis of tissues for deuterium were never associated with a complete study of the deuterium content of the water. Everything imaginable has been assayed for deuterium, but it has been compared to the average concentration of the deuterium and not to what the plants or animals, or whatever it was, received.

We are trying to make some rather exhaustive studies of tritium. What we have at the present time are averages on a series of measurements made on deer in the Southeast, in the Savannah River plant regions, and we have not found any concentration of tritium which exceeded the average of a large number of ambient water samples of tritium or of vegetation samples of tritium; but again that is an average.

It is the same situation as this double labeling where you have different molecules labeled; this is not convincing evidence, but we are running more of these in which the tritium intake is very carefully monitored over a period of time to see if any tissue accumulates more than the ratio in water between protium, deuterium, and tritium. I think really good experiments of that kind have not been done.

Dr. Dayton: If you will allow me to show one slide, perhaps it will answer the question.

$T_{1/2}$ Phenobarbital

Dose mg/kg	$T_{1/2}$ (hrs)			C_0 mg/l
	^{14}C	^{13}C	^{12}C	
30	48	---	45	67
23	56	55	---	

Assay: Butler, JPET 111, 425 (1954).

This was an experiment (carried out in collaboration with Dr. J. M. Perel and Mr. D. Dawson) to look into this question. If Los Alamos can present two mice, we can present one dog. (Laughter) This animal was first given a tracer amount of phenobarbital- ^{14}C mixed with ^{12}C phenobarbital; we then measured the plasma level half-life of phenobarbital using the method of Butler. Two weeks later we gave the same animal phenobarbital- ^{13}C (prepared from 60% urea- ^{13}C) mixed with a tracer amount of phenobarbital- ^{14}C . Because there was stress, there was a change in the half-life. But since we used phenobarbital- ^{14}C as a means of comparison, you can see that there was really no change due to the substitution by ^{13}C .

Our aim in this is to collaborate with Dr. Goldstein to look into the question of binding of drugs, potentially carbon-13 phenobarbital, to human serum albumin, because here we might have a way of looking at which carbon is actually binding to the albumin.

Dr. Gillette: Where was it labeled? Was that not a site that was metabolizing in phenobarbital?

Dr. Dayton: Years ago J. M. Perel, C. Tauriello, L. Brand, L. C. Mark, and I made p-deutero phenobarbital; it was labeled exclusively in the position where one obtains metabolism. We observed no change in the half-life of the drug. Not only did we find that, but when we isolated the phenobarbital from urine, there was a drop in the deuterium content of the phenobarbital itself. There was no primary isotope effect.

Chairman Tolbert: A tremendous amount of information is needed in the toxicity problem. In the case of nitrogen-15 the only toxicity data I know of is that the molecular biologists have used it in 60% concentrations, to label bacteria, with no adverse effects whatsoever. There are isotope effects between carbon-13 and carbon-12 in these biological systems and there may be differences. Nonetheless, with three different biological systems that have already been studied and with the lack of any induction of toxic effects in these systems, one can assume that the kinetic isotope effects have probably been absorbed by feedback control mechanisms and were within the limits of the normal biological processes to handle. All of this does not negate the necessity for further work on this problem before clinical work is done, but should encourage research on a clinical level.

Dr. Tarlov: I would disagree with your summation to a certain extent, perhaps not seriously. I would propose that there are no data at the present time which would satisfy a board or even myself that these substances can be given to human beings at the present time. I would think that, since the general feeling here is that these substances have such enormous potential in clinical investigation (which we feel is important to the development of better health for our people), an important outgrowth of this conference might be the determination to set up an advisory board or a task force. Such a board would not only include some of the people who are here, but would also include some of the people from regulatory bodies and the federal government and others to develop the kinds of toxicity studies that are going to hold up and will be scientifically valid. This will help us in the speedy introduction of these isotopes and our application of them.

I think *ad hoc* studies of the sort we can all think about are really not going to do the trick, and I think the political and the social climate in the United States is going to get us into a lot of trouble unless we do this properly.

Chairman Tolbert: Perhaps some of the people here will explore your suggestion, perhaps through the National Academy of Science.

Dr. Levine: Dr. Tarlov has just said most of what I wanted to say following the comments made by Dr. Mirkin and Dr. Clark. What would happen with the new carbon-13 labeled compound? The expectation was that it would be submitted with an IND to the Food and Drug Administration, who would not respond in 30 days. Of course you must have the approval of clinical investigation by an experimentation committee. Many clinical pharmacologists tend to be on such committees. Probably there are a number of people on these committees here. I have been chairman of such a committee for the last couple of years, and I just want to say I can see no basis from any of the discussion here for any decision of any sort from such a committee. They couldn't make an informed decision. They couldn't instruct an investigator as to how he would get informed on the subject. I want to underscore Dr. Tarlov's suggestion.

Dr. E. C. Horning: I don't think the situation is quite as black as has been painted. There is a certain amount of basic chemical knowledge here. Theoretically, toxicity could be affected. One might want to test it, but there is no ground for believing that this might happen. It is a different situation. Don't you think that is true?

Chairman Tolbert: I agree there is no theoretical basis for toxicity. In fact, before these toxicity studies were made, we had many long discussions and could find no basis for such effects.

Dr. Borg: There is no theoretical basis for toxicity.

Dr. Berlin: There is nothing to support that context. Bob Weiger and I sat on a committee on review of research in man, and I sat as chairman of the medical board at the NIH. I helped draft the rules for these things. You cannot get away from the fact that there is a body of law in terms of federal regulations in the FDA administration that deals with the introduction of new drugs. They are not going to accept the theory. They are going to ask the doctor for the evidence that this material is nontoxic in humans. They are going to ask for the evidence that it is or is not toxic in several species of animals at several levels of administration. They are going to ask about acute and chronic toxicity studies.

Some of us who have been concerned with problems of radiation toxicity have been through this. We don't know the fine structure of the toxicity of many of the radiation sources we have, whether they be diagnostic or isotopic. At least there is some attempt to quantitate some of the radiation toxicity in the experimental animal.

In my own field in the National Cancer Institute, I don't think we can predict what is and what is not a carcinogen. Chromium is a carcinogen not predicted from theory. There are well-known carcinogens. I think we are dealing with a very critical problem here. Don Borg says there is no theoretical basis for it, and it is foolish to consider it, and he may be absolutely right; but we are going to have to face groups that will not accept theory. They want data. What are the data?

Dr. E. C. Horning: That is entirely correct, and I don't argue the point. Toxicity studies will be needed, but there is a difference between toxicity studies where toxicity is known, radiation hazard for example, and where there is no theoretical basis for this. In other words, it is not saying don't do them, because they have to be done. The question is how much do you do.

Dr. Berlin: You have to do it once to satisfy a regulatory body.

Dr. Borg: I am glad all of this is going to be total suspension of reason and each new agent is going to be treated as though it were some kind of green thing from another planet, about which we know nothing. Carbon-13 is a natural constituent of all carbon. There are some variations in nature because there are isotope effects, and organic systems do tend to distill out carbon-12. When you use something from chalk or sea-water you are giving more carbon-13 than when you give carbon from a source such as methane or ethane. If you integrate the total amount of carbon-13 you are giving, it is a large amount.

We don't enrich carbon-13 in any special way. We don't sequester it in secret fashion. We have the kind of isotope effect that is seen and noted and documented. There is quite a range of carbon-13, from methane which has the least carbon-13 to oceanic bicarbonate, and some limestone is richest in carbon-13. We know something about how nature handles large volumes of it.

Dr. Gillette: I think we are confused as to how we are going to use this compound. Chronic administration of a compound with a highly enrichment in carbon-13 may produce some toxic effects. If you are going to use it only once and have a relatively low enrichment of carbon-13, then I don't think you are going to get into trouble, because the isotope effect is minimal.

Actually, the experiments to show lack of toxicity are relatively simple, because there are going to be only one or two experiments and you are going to see whether or not the animal survives for the rest of its life. I think for the things that the clinician wants to use them for, the experiments can be carried out.

Dr. Hofmann: I think Dr. Berlin has committed a logical fallacy because he is talking about different kinds of compounds and we are talking about different concentrations of the same element. As clinicians I think we should consider benefit-to-risk ratio. This may be the critical thing. It seems to me the benefit of what we are doing diagnostically may be greater than the risk, as far as we can tell.

Dr. Clark: The last two speakers, I think, spoke to an important point which I left out, namely, that the degree of safety requirements is always related to the intended use.

Dr. Pollycove: If there are 20 kg of carbon in the body, there are about 200 g, or a little more, of carbon-13. Earlier this evening it was stated that for most single experiments 1 g or less of carbon-13 would be administered. Aside from the fact that there is no theoretical basis,

to expect any toxicity we are only adding something on the order of 0.5% or 1% of the carbon-13 already present in the body. No matter how you look at it, it is hard to believe that this is a real danger.

Dr. Berlin: The theory is fine, and I believe it, but I don't represent a regulatory agency. What are they going to ask you? The argument may be logical or it may not be logical, but the people who make these determinations are going to ask you what acute and chronic toxicity is at various dose levels. This is standard practice.

Chairman Tolbert: I would like to refer to Table I, hoping to summarize the information from the conversations this afternoon.

TABLE I
ANALYTICAL METHODS FOR CARBON-13

Analytical Method	Gross Sample	Contained ^{13}C	Precision or Sensitivity
ESR	1-10 μg with 10^{12} spins		10% of nat. abundance
Mass spectrometry	0.1 - 100 ng		0.01%
^{13}C NMR	200 + μg	2 μg	0.1%
Proton NMR	10 - 50 μg		
Micro wave	1 mg		0.5%
IR Abs. (conventional)	~ 1 mg		0.5%
IR Raman		10 mg	
IR Fluorescence			
Density gradient	1 mg	3% enrichment	
Nuclear reaction		nanogram range	
IR Rotational Abs.			100% of nat. abundance

All the methods by which one can detect carbon-13 that I know of are on this list. It indicates some of the strength of the carbon-13 isotope because this great diversity of detection methods increases versatility in experiments. I would like individuals who are experts in these methods to add to this list the minimum gross sample size per assay, the contained carbon-13, the sensitivity, or sensitivity in percent of natural abundance or in terms of signal-to-noise ratio. (Following this, there was an attempt by the audience and chairman to fill in the Table. The figures given are working guesses from the participants.)

Chairman Tolbert: In finishing the discussion on instrumentation, I would like to conclude with the observation that problems in this area are at present instrument complexity as well as instrument sensitivity. This is a very critical problem on a clinical level. Sample handling in a biological study is another problem and the number of samples that one can analyze is critical. I have great faith that when interesting clinical applications are developed, these can be solved, and I foresee, with this wide variety of phenomena available, adequate developments will be made.

There is a tremendous need for development and this requires money. However development of instruments in isolation from the actual experimental context is also often futile. I would hope the instruments will be developed, as Dr. Klein is developing his, in cooperative programs with the biologists.

Dr. Goldstein: It might be pertinent to comment that keeping track of the current level of solid-state developments would help. You can now get a solid-state infrared emitter for a few dollars, from which you can design an infrared absorption instrument to operate monochromatically. If you are trying to develop a standardized instrument for a given purpose, it is conceivable that such could be put together in any reasonably well-equipped installation for maybe \$1,000 or \$2,000. With the sensitivity now available it could have real application. These devices are coming out so fast that it would be foolish not to keep track of them.

Dr. Borg: There is a modification for fluorescence IR using a non-dispersive infrared resonance. It looks like a very attractive way of measuring carbon-13 enrichments in CO₂ gas.

Dr. Klein: I want to add to what you were saying and make a plea to people not to develop a unique instrument. It is very, very easy to build a unique instrument at great cost and of great splendor. This is really not the direction we have got to go because only a limited number of these instruments can be built and it will not get this field going. The more general, widely-applicable, and less expensive instruments are, the better the use that will be made of them.

Dr. Watson: One point on the mass spectrometer. The lower limit might safely be brought down to 0.1 ng. This is dependent on the compound type; for methyl stearate we can get a discernible peak going down to 100 pg.

Another point that has not been mentioned is the purity of labeling. Can one get 99.9% carbon-13 labeling with one carbon? This is particularly important if one tends to look at these low levels of compound using a carbon-13 drug, let's say, a carbon-13 labeled drug as an internal standard for mass spectrometry. We would want to have such a standard that would be 99.99% labeled. I don't think we should just accept things that we now see in catalogs which are of the order of 90% labeling. Let's not be satisfied with that first try.

Chairman Tolbert: There is an interesting problem associated with the 90-95% carbon-13 which Dr. McInteer is now producing. In the distillation process the $^{12}\text{C}^{18}\text{O}$ distills with the $^{13}\text{C}^{16}\text{O}$ and the dilution of the carbon-13 by the former is such that one is limited by the distilling columns to approximately 95% enrichment. In order to do better it is necessary to scramble the carbon and oxygens without ruining the purity of the CO . One does not yet know exactly how to do this.

Dr. McInteer: I don't believe there will be great difficulty in going to the higher concentration if there is any need for it. Thus far we have not found a real requirement. As a matter of fact, we are putting in a special little column so we will be able to turn out 100% or 99.99% rather than 90%. I think it will go just fine. It is just a matter of getting around to it.

Chairman Tolbert: I would like to have a couple of suggestions from the floor about experiments to be analyzed. Does anybody have an experiment to propose?

Dr. DeGrazia: I often get questions from our pediatrics group about protein turnover values, clearance rate of albumin, for example. It would be very nice to have a method to do this.

Chairman Tolbert: May I suggest an experiment? You are actually asking about the blood albumin turnovers; is that right? Let's assume I would label the blood albumin with an essential amino acid, say, lysine or arginine or any of these others, withdraw a sample, store it until general carbon-13 labeling had decreased in the body, and then return it to the pool. Could I make the measurements?

Dr. Klein: I think recycling of the essential amino acids is a problem. That is why arginine, labeled with nitrogen-15 in the guanido group, is so good. It is essential, but that portion with the label returns to the nitrogen-pool, is washed out, and when you recycle the arginine you don't have re-utilization of the label.

Dr. Tarlov: I am interested in normal physiological functions. I have a fundamental philosophy that the limitation to better health is not the need for better diagnostic tools but more complete understanding of physiology.

One of the problems I have been interested in, which I have taken as far as I can now because of the limitation of radioactive isotopes, is the cell membrane turnover, and particularly the relationship of protein to lipid in the membrane. I used the red cell membrane as my model. We have done a lot of work in the rat on this particular subject, and it turns out, for example, that among the single class of compounds, known as sphingomyelins, there are six different species, each chemically different, and each having a different rate of turnover. This implies, I believe, a different association with neighboring carbohydrate and protein in each case.

It takes a lot of isotope to do a study like this, because the lifetime of the rat red cell is 70 days. With the quantities of radioactive isotope that we now use, we have detected some toxicity in our rats, notably a momentary depression of the bone marrow activity. We have used tritium and phosphorus-32 and carbon-14. I think it is important that these studies be done on humans. They are impossible to do at the present time because of the safety questions. I am thinking that a stable isotope, if I were satisfied that it was a safe thing to do, would provide us with a new tool for studying membrane turnover, cell turnover, and metabolite production in the human being.

REPRODUCTIVE PHYSIOLOGY

Chairman: L. J. Roth

APPLICATION OF STABLE ISOTOPES TO STUDIES IN REPRODUCTIVE BIOLOGY

Uwe E. Freese

PERINATAL BILE ACID METABOLISM

Roger Lester

APPLICATION OF STABLE ISOTOPES TO STUDIES
IN REPRODUCTIVE BIOLOGY

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ABSTRACT

The exploration of the intrauterine environment during pregnancy is complex, difficult and for obvious reasons, frequently prohibitive. In view of the ever-increasing and pressing needs for such undertakings, the application of stable isotopes can be invaluable and their usefulness must be explored without delay. Uterine and placental hemodynamics, transplacental transfer, fate and effects of drugs on the fetus are examples of problems to which a solution may be forthcoming through the use of stable isotopes.

In recent decades the use of labeled substances has increased dramatically. The reasons are obvious. Labeled compounds are not only detectable in body regions, organs and circulatory systems, but at the cellular and subcellular level. Once a certain degree of expertise had been obtained in handling these isotopes, investigative work *in vivo* was cautiously applied to *in vivo* studies including those in man. The celebrated example is ^{131}I for the study of thyroid function. This innovation was of major significance because purely morphological and histochemical studies were supplemented and broadened to more or less physiological function observations. Once introduced into medicine, practical aspects of great significance clearly emerged and the therapeutical application of isotopes quickly became a reality. Today metastasizing cancer of the thyroid, particularly in cases where the disease has progressed beyond the reach of the knife, is being treated successfully with therapeutic dosages of ^{131}I . One could cite other examples to demonstrate the great impact this development has had on exploratory and therapeutic medicine.

The rapid advancement in the development of more sophisticated instrumentation for analytical dissection of biological processes, requires definitions in greater detail of physiological as well as pathological developments in the bodies of experimental animals as well as in the human. While some progress has been made in these

areas, quicker advancements are considerably hampered mainly because isotopes are, or are potentially, harmful to the various cell systems. For example, one large class of organic compounds of physiological interest are the N-methyl-amines, however, a small amount of the radiocarbon which arises from the oxidation of the N-methyl group, appears to enter the nucleic acid pool. This then makes it unsuitable for lengthy studies in patients.

The development of isotopes with a short half life, for example ^{123}I versus ^{131}I , has solved only part of the problem and added new ones, i.e., the need for particle accelerators which raises difficulties in regard to economic and logistic problems.

Another example is technetium-99 which is used for placental localization studies. Incorporation into an appropriate radio-pharmaceutical is necessary, the production of which is currently almost completely exempt from FDA controls. All indications are, however, that more stringent regulations will be imposed, resulting in tedious and time-consuming practices at the local level and hospitals in turn may prefer to use centralized regional radio-pharmaceutical units.

Current developments in medicine indicate that our research for a better and deeper understanding of biological processes is going to be profoundly influenced by over-riding concerns for the safety of our investigative methods. Concerned legislators and citizens are going to take a hard look at well-established practices in clinical research. While all of us share in their concern, as evidenced by the establishment of clinical research review committees, we must realize that many forthcoming restrictions will not be to our liking. Accordingly we must come up with something more refined and less potentially harmful to the patient.

A concerted effort, such as is exemplified by this symposium, to explore the possibilities of stable isotopes in biological research is an excellent means by which apparently insurmountable problems can be solved. Those of us who are doing research in reproductive biology are constantly confronted with perplexing questions relating to the harmfulness of whatever methodology is applied to explore the implanting blastocyst, the developing and differentiating embryo and the growing fetus. For this and other reasons we should be, and are, so extremely interested in the availability of stable isotopes. For obvious reasons, it is exceedingly difficult to explore in the human, developmental processes *in utero* at any time of gestation. Primarily because of this, an incredible amount of literature has accumulated reflecting the efforts of researchers from virtually all disciplines to extract from all conceivable animal species that information which we wish we could obtain directly from the human. But the ever-present fear that permanent changes may be inflicted through interference with an established pregnancy or a normally-developed ovum in the ovary, temper our efforts.

I would like to offer this morning, a few thoughts which reflect on some of the many problems waiting for a solution. As an obstetrician and gynecologist whose research interest is in placentology, I would like to use the placenta for this discussion. As all of you know, this intriguing and fascinating organ is a part of the fetus and develops from a tiny structure measuring, at the time of implantation a few microns, to a mature organ weighing, at term, about 500 g with a total surface for exchange between mother and fetus of approximately 10 - 14 square meters. It is connected to the fetus through the umbilical cord and to the mother through cell columns which are implanted in the wall of the uterus. It is subdivided into lobes and cotyledons which in turn are composed of innumerable small branch-like units or villi. The cord, the cotyledons, and villi are supplied with blood vessels containing fetal blood which pass from the fetus through the cord into the tiny capillaries located in the most peripheral villi of the placenta. This vascular system is a closed system. On the maternal side, there is the uterine wall into which the cell columns of the placenta have penetrated and tapped maternal arteries and veins. Thus, maternal blood leaves the circulation, running through tiny spaces, in between the innumerable villi before returning into the maternal circulation through maternal veins. This mechanism for approximation of the maternal and fetal blood is designed for the purpose of exchange of nutrients, waste products, and gases between the mother and the fetus. The only separating membrane between the mother and fetal blood is the lining of the placental villi and the fetal capillary. This membrane averages about $3\frac{1}{2}$ to 5 μ . Recent data, based upon biochemical and electron microscopical observations clearly indicate that several mechanisms are involved in the transfer of substances and gases from the mother to the fetus and vice versa. These are:

1. Simple diffusion in accordance with Fick's Law (O_2 , CO_2 , water electrolytes, most substances with molecular weight of 500 or less).
2. Facilitated diffusion (faster than according to Fick's Law, deviating at high concentrations).
3. Active transport involving expenditure of metabolic energy.
4. Pinocytosis and micropinocytosis.

Biochemical, electron microscopic, and to a limited extent, isotope studies, have allowed us to identify these mechanisms. There is a pressing need to re-evaluate the recorded findings and to start without delay, the exploration of the many unanswered questions in this particular field. I would hope that stable isotopes will permit us to move ahead.

Of course, transfer processes require an intact, uninhibited circulation of blood through both circulatory systems, i.e., that of the placenta and the uterus. Measurements of uterine and placental blood flow have been attempted many times with a variety of ingenious

devices. Most of these approaches require extensive surgical procedures for insertion of flow meters and mechanical or pharmacological alterations of the vascular systems. These are all basically very undesirable intrusions that often compromise the physiological state of the experimental animal. In this context we are reminded of the classic studies of Plentl and associates who explored with isotopes, ^{42}K , and ^{24}Na , the production and transfer rate of amniotic fluid in human pregnancies. These were elegant and revealing experiments but they were limited in scope because of the powerful radiation and short half-life of ^{42}K . I can foresee a vast extension of this work including the very important aspect of uterine hemodynamics, normal and pathological, through the use of stable isotopes.

A very large share of our ante- and intrapartum fetal losses are due to alterations in the utero-placental circulation, particularly in the group of high-risk patients, i.e., those with high blood pressure diabetes mellitus and renal-vascular disease. The large losses in prematurely-born infants is a sad fact about which we can do very little at this time. Alteration in the uterine circulation may well be the offender responsible for the onset of early contractions and expulsion of the premature infant. We are more than anxious to find out whether or not this is so. Likewise we would like to know the fate of the drugs which we know can negate these unfavorable circulatory disturbances of the uterine vascular bed. Where do these drugs act, to what extent are they crossing the placenta and to what extent do they affect the fetus? Localization studies with isotopes not harmful to the gonads of the mother or fetus would be ideally suited in the attack and solution of some of these problems.

In recent years, much emphasis has been placed on the adverse effects of malnutrition, drugs, and other chemicals on the developing embryo and the functional integrity of the placenta. It is surprising to find, as one explores these complex problems, how little we know about the normal aspects of feto-placental development, about transplacental transfer, about the biochemistry of intrauterine nutrition, let alone the abnormal aspects. There surely is no place for complaints as to why so little is known and as to why so little has been done. There is indeed, a lot of information about the principles of intrauterine nutrition, transfer of nutrients and gases, but we have not been able to find the proper tools to test, in the human, what has been observed and extracted from *in vitro* and animal experiments. Again, it seems to me that the application of stable isotopes should help to overcome this dilemma.

Studies with isotopes like other methods, involving the placenta have their limitations. For example, the placenta conceivably can make a selection between the natural element and the isotopes, and this isotope effect could be a problem, particularly in experiments with stable isotopes where the concentration of the isotope can reach significant proportions. However, Plentl studied the isotopic

effect with D_2O and THO injected into amniotic fluid and found practically no difference.² Likewise, the possibility of an exchange reaction between the tagged atom and the corresponding stable atom exists. Since this problem is usually restricted to, for example, labeled proteins and red cells which are liable to hemolysis, no real difficulties should be expected. The placenta which serves the fetus *in utero* as lung, kidney, intestinal tract, and important producer of hormones, should be an ideal target for extensive explorations using stable isotopes.

At this point in time, a more fruitful exploration of this organ could be undertaken if:

1. Stable isotopes become more readily available.
2. Greater access is gained to human placentas and fetuses through the rapidly increasing number of therapeutic abortions.

A study of placental permeability could be divided into three categories:

1. Qualitative analyses on what is transferred.
2. Quantitative analyses on what is transferred and how fast.
3. Localization studies on where and how it crosses the placenta.

Whenever a fetus or a normal newborn or even the uterus become available, the same basic analyses could be obtained.

Time does not permit us to explore in detail the formation, transfer, and metabolic incorporation of the various nutrients, excretory, chemical, and pharmacological functions which sustain or adversely affect the life of the fetus. But I would like to point out a few of the pressing problems awaiting solutions. Proteins for the fetus appear to be derived mostly from amino acids circulating in the maternal plasma. The placenta permits their rapid passage, appears to be selective and maintains a concentration gradient from the fetus to the mother. We don't know where in the placenta these amino acids cross, which amino acids are blocked from crossing, and absolutely nothing is known about the transfer of peptides and polypeptides. Nothing is known about the actual fetal requirements for intact proteins and how much the fetus is able to synthesize. Even though the fetus is supplied with antibodies by the mother, it is unclear to what extent, if any, it can produce its own, particularly during the latter part of pregnancy.

Carbohydrates are readily available to the fetus from the maternal pool. However, the transfer mechanism is not sufficiently explained by simple diffusion. Stereo-specific transport mechanisms must be involved in view of different transfer times of sugars with identical molecular weights. The role of placental glycogen, which falls progressively during pregnancy, has not been explained.

Lipids, their origins, synthesis by the fetus, and their contribution to the caloric needs of the fetus are poorly understood and urgently require clarification.

The transfer of minerals has been investigated with the use of isotopes, but has been limited to those that were readily exchanged or were restricted to animal experiments. For some of these substances, an uni-directional flow has been advocated without demonstrating what regulatory mechanisms prevent the fetus from overaccumulation and possible intoxication. This is a most important area to be investigated, particularly in regard to sodium transfer in connection with toxemia of pregnancy.

And finally, the study of drug transfer and placental and fetal distribution is of greater importance today than ever before. The greater availability of human conceptions offers a unique opportunity to begin, without delay, in-depth studies.

I hope that these short remarks about the utero-placental hemodynamics and placental transfer have identified for you the numerous missing links in our understanding of intrauterine growth and development, normal and abnormal, and how urgently we need the proper tools to close these gaps. It is hoped that our discussions here will generate among us the concern, interest, and desire to work together on these problems and that we may confidently look forward to a fruitful cooperation between all disciplines, no matter how seemingly remote the contact between nuclear chemistry and physics and medicine may have been prior to our common effort here.

PERINATAL BILE ACID METABOLISM*

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In the adult, bile acids play a central role in the absorption of lipid by promoting the lipolysis of ingested triglyceride, and dispersing the products of lipolysis within the intestinal lumen. Thus, lipid is converted to a potentially absorbable material with increased access to the intestinal epithelium. Despite the intimate relation between bile acid and lipid absorption established in adults, virtually nothing is known about bile acid metabolism in the newborn. This deficiency is especially notable because lipid is a major caloric constituent of milk, and recent studies have established marked variation in neonatal fat absorption. The normal, (and in particular premature) newborn infants, maintained on a standard formula diet fails to absorb ingested fat completely and experiences steatorrhea. Under these circumstances, caloric intake may become sub-optimal. This, in turn, is of especial importance since recent studies suggest that caloric deprivation in infancy may adversely affect neurologic and general body development. Previous studies of the failure of fat absorption in newborn infants have primarily employed the metabolic balance technique, and have emphasized the comparative composition of fat or fatty acid in milk or synthetic formulas. The relation between bile acid and lipid has largely remained unexamined. We have, therefore, undertaken a study of neonatal bile acid metabolism, beginning by investigating the developmental state in the fetus just before birth, and then examining the state of bile acid metabolism in the newborn infant.

Fetal studies were performed in collaboration with Dr. Benjamin T. Jackson, Department of Surgery. The "marsupializing" technique of intrauterine surgery devised by Dr. Jackson (1) permits the insertion of jugular vein, carotid artery, and bile duct catheters into a near-term dog, sheep, or monkey fetus with minimal disturbance of fetal homeostasis, and with minimal loss of amniotic fluid. Thus, ports for

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This work was supported in part by U.S.P.H.S. Grant AM09881 and by U.S.P.H.S. Career Development Award AM12,127.

intravenous infusion and for blood sampling are provided and fetal bile can be collected continuously. After insertion and externalization of the catheters, the fetal, uterine, and maternal wounds are closed and the mother is maintained under light anesthesia. Measurement of fetal blood pressure, heart rate, ECG, blood pH, and blood gases have established that the catheterized fetus is supported under essentially physiologic conditions.

In initial studies (1), near-term thoroughbred beagle dog fetuses were prepared *in utero* with vascular catheters only. An intravenous infusion of a tracer quantity of a representative labeled bile acid, 24-¹⁴C-cholic acid, was administered to the fetus. The mother was prepared with ligation of the cystic duct, gall bladder aspiration, and common bile duct cannulation. The maternal bile was thus collected during study. Since bile acid is rapidly cleared from the plasma and excreted by the adult liver, bile acid crossing the placenta from fetus to mother would be found in maternal bile. Thus, counts in bile acid excreted by maternal liver become a measure of placental transfer. At the end of 6 hr of infusion plus a 4 hr post-infusion recovery period, the fetus and mother were sacrificed, and the distribution of counts was determined. The results of three representative studies are shown in Figure 1. At 10 hr, the bulk of the infused 24-¹⁴C-cholic was cleared from the fetal plasma by the fetal liver. Three - 10% remained in the fetal liver and 60 - 70% was excreted in fetal bile and recovered from the fetal gall bladder and intestine. Placental transfer was minimal, and total recoveries equalled 70 - 80% of the infused dose.

In order to characterize the kinetics of fetal hepatic excretion, a second group of animals was prepared with fetal bile duct catheters and again infused with tracer amounts of 24-¹⁴C-cholic. The rate of appearance of 24-¹⁴C-cholic in fetal bile was determined, and this was compared to results of comparable studies in nonpregnant adults. As can be seen in Figure 2, in the nonpregnant adults, a constant maximal excretion rate was obtained within 1 - 1½ hr and reached 96% (mean value) of the infusion rate. Thus, by the end of infusion at 6 hr, the adults excreted 86% of the infused 24-¹⁴C-cholic acid, virtually all in the form of ¹⁴C-taurocholic acid. The general configuration of fetal excretion was similar, but the maximal excretory rate was attained only after 3 - 4 hr of infusion, and was only 83% of the infusion rate. Therefore, only 58% of the dose was excreted at 6 hr, again in the form of ¹⁴C-taurocholate. Thus, it can be seen that the fetal hepatic excretory mechanism, while resembling that in the adult, has distinctive properties.

Fetal-adult comparisons have been defined in additional studies (2). Fetal dog gall bladder and hepatic bile contain the two bile acids synthesized and excreted by the adult liver, cholic acid and chenodeoxycholic acid. When the bile acid precursor, 4-¹⁴C-cholesterol, was administered to 4 fetuses, 1.1 - 4.7% of the radioactive dose was incorporated into fetal cholic and chenodeoxycholic acid over 36 - 53 hr (3). While some of the 4-¹⁴C-cholesterol crossed the placenta and was metabolized to bile acid by the maternal liver, the specific activity of the maternal bile acid was less than 1/200th of the fetal bile acid. Thus, the mother could not be the source of the

labeled fetal bile acid, and it was established that the fetal liver, like the adult, is capable of bile acid synthesis. Of additional interest was the finding of deoxycholic acid in endogenous fetal bile (2). Deoxycholic acid is a "secondary bile acid," made by the intestinal bacterial dehydroxylation of cholic acid. Since the fetal intestine is bacteria-free, fetal deoxycholic acid must accumulate in the fetus, either by fetal hepatic synthesis through pathways other than those available in the adult liver, or by maternal-to-fetal transfer. 4-¹⁴C-cholesterol, administered to the fetus, was not incorporated into fetal deoxycholate, but 24-¹⁴C-deoxycholate administered to the mother was found in fetal bile. Thus, fetal deoxycholate derives exclusively from the mother, and the two primary fetal bile acids probably arise both from fetal synthesis and maternal-to-fetal transfer.

The bile acid pool size in the fetal dog is much smaller than the adult's when compared on the basis of body weight: 119 μ moles/kg versus 248 μ moles/kg (2). This contrast is even greater when the fetus and adult are compared on the basis of body surface area or liver weight. Similarly, a comparison of gall bladder and hepatic bile acid concentration establishes that the fetal gall bladder concentrates bile with only approximately 1/3 adult efficiency. If these forms of fetal immaturity persist in the newborn, lesser amounts and lower concentrations of bile acid would be available in the neonatal intestine to promote lipolysis and lipid absorption. As in the adult, bile acid is excreted into the fetal intestine, reabsorbed and returned to the liver, and re-excreted by the liver. Two characteristics of the fetal "enterohepatic circulation" however, are notably different from the adult. Our initial studies suggest that considerable reabsorption of taurine-conjugated bile acid occurs in the fetal jejunum; whereas, in the adult, reabsorption of the taurine conjugates is limited to a more distal point in the terminal ileum (4). The intestinal portion of the enterohepatic circulation is thus short-circuited. If proximal reabsorption persists in the newborn, the net effect would be to limit the availability of bile acid for the dispersion of fat at more distal intestinal sites. In addition, the fetal enterohepatic circulation of bile acid differs from the adult because of the presence of portal-systemic shunts in the fetus. In the adult, all of the blood perfusing the intestine passes through the hepatic vasculature via the portal vein before reaching the heart and the systemic circulation. Bile acid in portal blood is efficiently cleared by the liver, and little bile acid absorbed from the intestine escapes peripherally. In the fetus, some of the portal blood by-passes the liver through an anatomic fetal by-pass, the ductus venosus. Moreover, there are suggestions in the literature that blood perfusing the fetal liver does not come into as intimate contact with the fetal hepatic parenchymal cells as in the adult (5). Thus, bile acid absorbed from the fetal intestine may by-pass the fetal liver entirely, or may perfuse the liver and escape past hepatic parenchymal cells into the systemic circulation (1). The fetus has, in effect, an "entero-systemic-hepatic" circulation of bile acid. The ductus venosus closes functionally shortly after birth. however, the time it

takes the newborn to gain an effective hepatic parenchymal cell perfusion and, with it, effective plasma bile acid clearance, remains unknown.

If comparable defects in bile acid metabolism could be demonstrated in the human fetus, and shown to persist through birth and into the neonatal period, it would become possible to begin to comprehend the mechanism of neonatal lipid malabsorption and steatorrhea. Each of the forms of "immaturity" demonstrated above would lead to less efficient bile acid metabolism, and make bile acid less available for the lipid interactions which promote lipolysis and lipid absorption. It has, in fact, been possible in preliminary studies to demonstrate that primate (Rhesus monkey) fetal bile acid metabolism closely resembles that of the dog fetus; having, if anything, a greater degree of immaturity as evidenced by a greater dependence on placental bile acid movement (6). On the other hand, until recently it has not been possible to perform comparable studies in newborn infants. Since there was no question of employing radioactive isotopes in apparently healthy neonates, exploration of bile acid metabolism in premature and newborn infants using metabolic tracer methodology was not possible.

The introduction of stable, non-radioactive isotopes for use in metabolic studies, complemented with sensitive techniques for the measurement of stable isotope ratios, has opened the investigation of human perinatal metabolism to tracer methodology (7). While the preliminary investigations of neonatal bile acid metabolism described in brief below, are intended primarily as a demonstration of the feasibility of study, it is clear that it is now possible to approach a variety of problems related to infant physiology, nutrition, and borderline disease states which were previously unapproachable.

In order to evaluate neonatal bile acid synthesis rate and pool size, 2,2,4,4- ^2H -cholic acid was prepared by enolization exchange and purified, according to the method of Hofmann, Szczepanik and Klein^{*} (8). Five mg of ^2H -cholic acid was administered via duodenal tube to three full-term normal newborn infants on the third day of life^{**} (9). The appearance of isotope in fecal bile acid and its rate of dilution were measured over the succeeding 5 days. Steady state conditions were assumed for the purposes of study. The assumption of a steady state cannot be proven because of the present limitations of

^{*} The author is deeply indebted to Dr. Klein and Dr. Hofmann for supplying the ^2H -cholic acid employed in these studies.

^{**} Permission for the performance of this series of studies was obtained from the institution's Human Investigation Committee. Consent for each individual study was obtained from the parent after complete explanation of the procedures of the test. In addition, in each case, consent was obtained from a physician-"patient advocate" not associated with the study.

the art, but weight changes for the infants were less than $\pm 5\%$ during the period of study. The three curves obtained are depicted in Figure 3. It is evident that isotope dilution occurred at a constant rate (linear regression analysis, $r = 0.92$ to 0.99). This establishes that cholic acid is excreted, i.e., that label is lost in feces, and that the excreted material is replaced by endogenously synthesized (unlabeled) cholic acid, i.e. that the ratio of isotopically labeled:unlabeled bile acid diminishes progressively over 5 days. The pool size is proportional to the ordinate intercept and is calculated from the formula (10):

$$\text{Pool} = \frac{\text{Dose Administered}}{\% \text{ Atoms Excess}(t_0)}$$

The range of pool size values 31 to 32 $\mu\text{moles/kg}$ or 494 to 570 $\mu\text{moles/m}^2$ body surface. The synthesis rate is proportional to the slope of the isotope dilution curve and the pool size, and is calculated from the formula:

$$\text{Synthesis Rate} = \text{Pool} \times K$$

$$K = \text{Slope of Isotope Dilution}$$

The range of synthetic rates equalled 9 to 12 $\mu\text{moles/kg}$, or 156 to 193 $\mu\text{moles/m}^2/\text{day}$. Establishing the basis for comparison with adult values is problematic; however, the most valid comparisons of energetic and metabolic requirements between infants, children, and adults are based on body surface. The infant values for pool size and synthesis are 1/3 to 1/2 published adult values (11) when compared on the basis of either body surface or estimated liver size. The results suggest that there is a deficiency of bile acid available for lipolysis and lipid absorption in the neonatal intestine, which may contribute to the development of neonatal steatorrhea.

The foregoing preliminary investigations are among the first tracer metabolic studies of metabolism in the normal newborn infant. The increasing availability of ^{13}C , the modification of mass spectrometer techniques for the detection of stable isotope at high dilution in small biologic samples, the introduction of low-cost, simplified modifications of these techniques for use in medical research laboratories, and the on-going collaboration between physicists, chemists, and medical biologists outlined at this meeting, promise to introduce a new era in the investigation of human fetuses and newborn infants. Our own investigation of neonatal bile acid metabolism will ultimately provide insight into the mechanisms of lipid nutrition in the newborn, the basis on which to devise techniques for the detection of abnormalities of neonatal nutrition, and ultimately, appropriate corrective therapy.

REFERENCES

1. Jackson, B. T., Smallwood, R. A., Piasecki, G. J., Brown, A. S., Rauschecker, H. F. J., and Lester R. Fetal bile salt metabolism. I. The metabolism of sodium cholate- ^{14}C in the fetal dog. *J. Clin. Invest.* 50, 1286, (1971).
2. Smallwood, R. A., Lester, R., Piasecki, G. J., Klein, P. D., Greco, R., and Jackson, B. T. Fetal bile salt metabolism. II. Hepatic excretion of endogenous bile salt and of a taurocholate load. *J. Clin. Invest.* 51, 1388 (1972).
3. Lester, R., Little, J. M., Greco, R., Piasecki, G. J., and Jackson, B. T. Fetal bile salt formation. Presented at the annual meeting of the Pediatric Research Society, May 1972. *Ped. Res.* 6, 1972 (in press) (abstract).
4. Smallwood, R. A., Lester, R., Brown, A. S., Piasecki, G. J., and Jackson, B. T. Fetal bile salt absorption. *J. Clin. Invest.* 49, 90a, (1970) (abstract).
5. Montagnani, C. A. Intrahepatic vascular pattern in the newborn infant. *Ann. N. Y. Acad. Sci.* 111, 121, (1963).
6. Smallwood, R. A., Lester, R., Piasecki, G. J., Little, J. M., and Jackson, B. T. Bile salt metabolism in the fetal monkey. Presented at the annual meeting of the American Association for the Study of Liver Diseases, November, 1969. *Gastroenterology* 58, 300, (1970) (abstract).
7. Klein, P. D., Haumann, J. R. and Eisler, W. J. Instrument design considerations and clinical applications of stable isotope analysis. *Clin. Chem.* 17, 735, (1971).
8. Hofmann, A. F., Szczepanik, P. A., and Klein, P. D. Rapid preparation of tritium-labeled bile acids by enolic exchange on basic alumina containing tritiated water. *J. Lipid Res.* 9, 707, (1968).
9. Watkins, J. B., Klein, P. D., Ingall, D., and Lester, R. Bile salt metabolism in newborn infants. Presented at the annual meeting of the Pediatric Research Society, May 1972. *Ped. Res.* 6, (1972) (in press) (abstract).
10. Hofmann, A. F., Schoenfield, L. J., Kottke, B. A., and Poley, J. R. Methods for the description of bile acid kinetics in man. In: *Methods in Medical Research*, Yearbook Publishers, Chicago, Illinois 1970, 149-170.
11. Vlahcevic, Z. R., Bell, C. C., Jr., Buhac, I., Farrar, J. T., and Swell, L. Diminished bile acid pool size in patients with gallstones. *Gastroenterology* 59, 165-173 (1970).

Figure 1. Final distribution of radioactive label after a 6 hour infusion of $24\text{-}^{14}\text{C}$ -cholate into near-term dog fetuses and a 4 hour post-infusion period. (Reprinted from *J. Clin. Invest.* 50, 1286 [1971].)

Figure 2. Excretion of radiolabel in bile after the infusion of $24\text{-}^{14}\text{C}$ -cholate in (A) fetuses and (B) nonpregnant adults prepared with biliary catheters. (Reprinted from *J. Clin. Invest.* 50, 1286 [1971].)

Figure 3. Turnover of 2,4- ^2H -cholic acid in 5 full-term normal infants. Rate of isotope dilution in bile acid recovered from feces after gastrointestinal administration of labeled cholic acid on the third day of life.

DISCUSSION

Chairman Roth: Discussion is now open.

Dr. Freese: I would like to make an additional comment. We have to conduct most of our experiments with regard to pregnancy in animals. Dr. Ramsey, at Carnegie, and Dr. Hall, at our laboratories, have finally arrived at the conclusion that the Rhesus monkey and the baboon are really so similar to the human, as far as the uterus and the placenta are concerned, and as far as the total relationship between these two animals and the human is concerned, that we should as of now, I think, try to abandon animal experiments which do not utilize these two species.

I would make a plea at this point that we get into the monkey business and use, for our experiments, the Rhesus monkey and the baboon exclusively because we do feel, without reservation, that we can apply the results in these two animals to the human condition.

Dr. Lester: I really have to agree completely. We know in our studies that the fetal dog does not behave in all respects in the same way as the monkey or the human. While we have some NIHers here, perhaps it is worthwhile adding that a researcher working in this area finds it almost impossible to obtain an adequate number or adequate source of pregnant monkeys for this kind of study. Perhaps the NIH ought to think about more extensive support for colonies of pregnant monkeys than is now available.

The second thought I would like to add is that if one wants to perform this kind of study, even if one thinks that the monkey resembles the human (and I think the Rhesus monkey is a good model for human studies), when all is said and done, such a study simply has to be validated in a limited number of human studies in order to be certain that the physiology or pathology that is analyzed and developed really applies to the human situation. Again, the use of stable isotopes in this field, in the study of humans, is an essential.

Dr. Gillette: In many instances the Rhesus monkey's metabolism is completely different from that of man. You cannot assume, therefore, that any mutagen or carcinogen effect seen in the monkey will be seen in the human.

Dr. Hofmann: There is one other aspect. As long as one is seeking unanimity from this point of view, I believe, not knowing much about it, that a fair number of drugs is metabolized by tissue systems as well as bacterial systems, and it seems to me very unreasonable to predict with any certainty that the bacterial flora of the primate will be identical to that of man.

Dr. M. C. Horning: There is something else to be said for monkeys as a model, and that is steroid metabolism in the newborn chimpanzee is very, very similar to that of the newborn human; and, as you know, the human newborn has an extremely interesting and very active steroid metabolism. The production averages 7 mg to 25 mg a day for a 3 kg infant. These are steroids that are not in the corticoid series at all. It is a different family. The chimpanzee does have the same kind of steroids, so in that respect it is a good model in the human.

Dr. Dayton: I would like to add to Dr. Freese's point. Therapeutic abortions would be useful but I think people should take advantage of anencephalics to study the placenta changes with time.

Dr. Freese: Yes, very much so. To find an anencephalic is really something. It is very, very difficult. Even out at the Cook County Hospital, where they are still delivering something like 20,000 patients a year, it is unusual, and there are various people who are looking for those. Usually it is a matter of who comes first gets it first.

I think we are more prone to extend some of our indications when it comes to therapeutic abortions, for example, when we decide as to what certain procedure has to be performed. There are very many patients who are likely to give their consent to have a complete hysterectomy performed rather than evacuation of the uterus and tubal ligation. I am talking about patients who are now 40 years of age, who already have twelve children at home anyway and are excellent candidates for cervical pathology. So, this is not being done with young people. There are good sound medical indications for these procedures. The material is available.

Dr. Mirkin: I thought it might be helpful to give some comments on the existing status of our knowledge in this area. There currently exists today data referable to perhaps eight or nine drugs in the human for which we have information on the fetal distribution. We have virtually no information on the placental kinetics, that is, the rate constants of transfer of drugs across the placenta.

It is turning out to be a very hazardous extrapolation to take data from other types of membrane systems such as rate constants of drugs across the blood-brain barrier or across the kidney tubule and applying that to the placenta. Those don't seem to hold up. Again, I re-emphasize the need for work in this area.

Even in the animals, there seems to be a paucity of data. Fifteen drugs have been analyzed in this respect, and there is some information available which may be useful.

Chairman Roth: I appreciate that remark very much, except that to compare the blood-brain barrier to the fetal barrier, is (I think), really far-fetched, because nobody knows really what the blood-brain barrier is.

Dr. Klein: I want to add a plea on behalf of the clinicians, that we not overlook the fact, that, in addition to the research which one can do on specific species, one very serious and important contribution that stable isotopes can make is in the diagnosis of defects such as biliary atresias which have been mentioned before--these studies that are concerned with defects in the patients which are not accessible by conventional means. I would single this out again because I think that it is equally important in the delivery of health care.

Dr. DeGrazia: I think medicine's problem is the lack of a strong dialogue between the people who are in the basic sciences and doing animal research--strong enough dialogue, I should say--and the people who are in the medical profession. What is needed is to take some of the information that already exists in the basic sciences and apply it to useful diagnostic and hopeful testing of treatment and perhaps even methods for treatment. This might be one of the reasons why the public is somewhat disillusioned with the rising cost of medicine and the results coming out.

What I am really saying is that some of these things ought to be applied, and promptly, to humans. I think much can be done. I think we are a little too frightened about applying some of our techniques to human investigation.

Chairman Roth: For a long time, I worried about the lack of dialogue between the basic scientists and the clinicians and I mentioned this to someone yesterday and he said, "Why don't you run a conference on that subject?" (Laughter)

Dr. Hofmann: I would like to disagree with both of the two previous speakers. We do have many diagnostic procedures, but in some areas we are grossly deficient. We have extraordinarily cumbersome diagnostic procedures. If anybody is sent to a clinic to find out if their intestine can absorb fat, they have to collect stools for 72 hr. This determination has not been improved since 1925.

The breath test I told you about yesterday is the first test we have ever had for looking at interaction between bile acids and bacteria, and this is just one of many tests. We need many more. I would further submit that I work with 700 physicians who are always very anxious to capitalize on any basic science discovery. As soon as a real advantage is apparent the work will explode.

The field of antigens, which is now being used to screen blood which transmits long-incubation hepatitis, is a field discovered 3 years ago, and I think there are now probably 1,500 papers on the subject. I admit the dialogue isn't perfect, but as soon as the application becomes the least bit obvious, I submit that the medical and clinical profession does move in.

Dr. Hurwitz: In the comments that were made before about diagnosis, these are usually situations in which you are dealing with a relatively unhealthy individual, in which the benefits versus the risks of the diagnostic procedure can be weighed. In these situations perhaps the need for the ultimate safe procedure is not as great as it is in the normal physiology situation in which we are exploring normal people who are not exposed to any risk to begin with. The question should not be as much for diagnosis as it should be for evaluating normal physiology so we can better understand what we are doing.

Chairman Roth: Thank you. That clarifies the statement very nicely.

STABLE ISOTOPES IN CLINICAL PHARMACOLOGY

Chairman: M. Bush

LABELING WITH ^{15}N OR ^{13}C FOR IDENTIFICATION
OF MASS SPECTRA OF DRUGS AND THEIR METABOLITES

*M. T. Bush, H. J. Sekerke, Jr., M. Vore,
B. J. Sweetman, and J. T. Watson*

THE USE OF MULTIPLE ION DETECTION IN DRUG METABOLISM METHODOLOGY

J. Throck Watson

USE OF STABLE ISOTOPES IN PHARMACCOLOGY -
CLINICAL PHARMACOLOGY

Daniel R. Knapp and Thomas E. Gaffney

CODING OF BARBITURATE SOURCES BY STABLE ISOTOPE LABELING
DURING MANUFACTURE

Mark A. Minton and Peter D. Klein

AN ANALOG MULTIPLEXER FOR MULTIPLE SPECIFIC ION DETECTION IN
A GAS CHROMATOGRAPH/QUADRUPOLE MASS SPECTROMETER SYSTEM

Donald J. Jenden and Robert W. Silverman

LABELING WITH ^{14}N OR ^{13}C FOR IDENTIFICATION
OF MASS SPECTRA OF DRUGS AND THEIR METABOLITES

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ABSTRACT

Labeling of a drug with a stable heavy isotope such that the ratio of heavy to light isotope is approximately 50:50 makes it possible to identify at a glance those mass spectra which are drug-related, by virtue of the unique twin-peaks produced by such pairs as ^{14}N : ^{15}N and ^{12}C : ^{13}C , etc. We have used the former isotopes for study of the metabolism of two unusual barbiturates and are synthesizing bufotenine- ^{13}C for similar studies.

The identification of a drug (extracted from biological materials) by GC or GC/MS is dependent on direct comparisons with the pure drug itself. For the identification of metabolites of the drug, however, reference material is often not available. The usually grossly impure extracted substances give many GC peaks and a laborious examination of the many corresponding mass spectra may be required before drug-relatedness of unknown products can be established. Some time ago it occurred to one of us that the magnitude of this problem could be minimized for any particular drug by synthesizing the drug with a heavy-light (stable) isotope ratio of 1:1 in a suitable locus (metabolically stable) so that the mass spectra of the metabolites as well as of the drug itself would (or should) contain twin peaks one mass unit apart, a phenomenon very rare under normal circumstances and thus capable of giving immediate visual characterization of a mass spectrum as drug-related.

A new barbiturate, of special interest to us, was the first compound we labeled in this way: 5-ethyl-5-tertiary-butyl-barbituric acid (1). The urea moiety was labeled with ^{14}C and at both nitrogens. The ^{14}N : ^{15}N ratio was adjusted to approximately 1:1. Examination in the LKB-9000 elicited from Dr. Watson the remark: "those (twins) can be seen from across the room." The twin peaks were two units apart since both nitrogens were labeled. Characterization of the metabolites of this substance is incomplete.

A re-investigation of the metabolism of 1-n-butyl-barbital (2) has been underway in this laboratory for the last couple of years. The compound is extremely lipid-soluble and a potent hypnotic. The structure is shown in Figure 1. To facilitate study of its metabolic fate (in rats) the urea moiety has been labeled with ^{14}C and with ^{15}N at the -NH position, by well-established synthetic reactions. The ^{14}N : ^{15}N ratio at this position has been adjusted to approximately 1:1 by dilution with unlabeled drug.

The compound is readily volatile and quite stable. In the LKB-9000 it gave the fragmentation pattern shown in Figure 1. The molecular ions are not prominent but are unequivocal at m/e 240 and 241. The major twin peaks at 185-186 are equivalent to m-55.

The major metabolites of this drug have been tentatively identified with the aid of the double-label (3). An alcohol (suitably derivatized) and a ketone both give mass fragmentation patterns containing several twin peaks. Details will be reported in the near future (Figure 2,3).

We are presently synthesizing bufotenine-2- ^{13}C to aid in the identification of its minor metabolites. Its major metabolites in the rat have been characterized (4). In this molecule the $-\text{N}(\text{CH}_3)_2$ group is metabolically unstable (over 50% of the dose is converted to 5-hydroxyindoleacetic acid in man and almost as much in the rat) thus making it undesirable to use the ^{15}N label.

A similar potential use of "isotopic doublets" has been noted for the natural chlorine isotopes in chlorpromazine (5). The study of the metabolism of nortryptiline labeled with "isotopic clusters" of m, m+2 or m+3, achieved with suitable amounts of ^2H and ^{15}N at particular loci, has been facilitated in this way (6,7).

This work was supported by NIH Grants NS-3534 and GM 15431.

REFERENCES

1. H. J. Sekerke, Jr. *Fed. Proc.* 28, 290 (1969).
2. M. T. Bush and T. C. Butler. *J. Pharmacol. Exp. Ther.* 68, 278-283 (1940).
3. M. Vore, N. Gerber, and M. T. Bush. *The Pharmacologist* 13, Vol. 2, 220 (1971).
4. E. Sanders, and M. T. Bush. *J. Pharmacol. Exp. Ther.* 158, 340-352 (1967).
5. C.-G. Hammar, Bo Holmstedt, and R. Ryhage, *Anal. Biochem.* 25, 532-547 (1968).
6. D. R. Knapp, T. E. Gaffney, R. E. McMahon, and G. Kiplinger. *The Pharmacologist* 13, Vol. 2, 220 (1971) *J. Pharmacol. Exp. Ther.*, *in press*.
7. D. R. Knapp and T. E. Gaffney, "Use of Stable Isotopes in Clinical Pharmacology." This seminar, p. 249.

Figure 1. Mass fragmentation of N-n-butyl-barbital, by direct probe in the LKB-9000.

Figure 2. Mass fragmentation spectrum of "Metabolite B."

Figure 3. Mass fragmentation spectrum of TMS-N-methyl derivative of "Metabolite A."

THE USE OF MULTIPLE ION DETECTION IN DRUG METABOLISM METHODOLOGY

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ABSTRACT

In multiple ion detection (MID) the mass spectrometer serves as a gas chromatographic detector which has high specificity, adjustable to the characteristic fragmentation pattern of the compound of interest. Examples are presented here which demonstrate the qualitative usefulness of MID in developing new vapor-phase methodology, quantitative applications involving stable isotope-labeled compounds both as internal standards and as carriers for polar, endogenous molecules, and, finally, the advantages of on-line data acquisition with a small laboratory computer.

Quantification of drugs and drug metabolites at physiologically significant levels in body fluids often places high demands on analytical methodology. Adequate sensitivity and specificity can sometimes be demonstrated by gas-liquid chromatography (GLC) with electron capture detection (ECD) in the analysis of synthetic mixtures of standards, but this technique can also be easily overwhelmed by "contaminants" when applied to realistic biological samples. While GLC-ECD is desirable because of its simplicity, a more specific technique such as gas chromatography-mass spectrometry (GC-MS) with multiple ion detection (MID) is useful in guiding the development of preliminary purification which must necessarily accompany the more conventional means of quantification in vapor-phase methodology.

The high specificity of MID is achieved by adjusting the GC-MS to monitor the ion current at only 2 or 3 m/e values; the MS detector will then respond only to those substances in the GC effluent which can generate these specific ions. Operation of the GC-MS in the MID mode involves monitoring selected ions rather than scanning the complete mass spectrum.

The results of one semi-quantitative use of MID are represented in Figure 1 which shows 2 analytical traces recorded in parallel from a single injection of a urine extract analyzed by GC-MS for

normetanephrine (NMN) as the pentafluoropropionyl (PFP) derivative. The top panel of Figure 1 is a profile of total ion current (TIC) which is comparable to a data trace from a conventional flame ionization detector on a GLC. The bottom panel (same time scale) is an oscillographic record of MID in which the GC-MS is focused alternately on m/e 445 and m/e 458 which are principal ions in the mass spectrum of NMN-PFP (Figure 2). None of the peaks in the upper panel correspond to the expected retention time for NMN-PFP; in fact, no discernable peak is indicated at the expected retention time of 3.7 minutes. On the other hand, the bottom panel showing the MID data record, indicates the presence in the effluent of a substance having a retention time of 3.7 minutes which produces ion current profiles of m/e 458 and 445 in the ratio of 2:1 as expected from NMN-PFP. These data qualitatively confirm the presence of NMN-PFP in the biological matrix (1) which, of course, required further purification before it was amenable to analysis by more conventional techniques such as GLC with ECD. Since the introduction of MID (2), other qualitative uses (3) and, more recently, quantitative applications (4-7) of the technique have been reported.

The interest in prostaglandins in our laboratory has resulted in a comprehensive evaluation (8) of a variety of derivatives of prostaglandins from the standpoint of gas chromatographic behavior, thermal stability, polarity, ease of formation, and fragmentation pattern. The bis-acetate of the methyl ester, methoxime derivative of prostaglandin F_2 is particularly suitable for quantitative work because upon fragmentation, the majority of the ion current is carried by m/e 419 which is generated by the loss of the elements of acetic acid (60 atomic mass units) from the parent molecule. Measurement of this most abundant ion should permit better overall sensitivity. For purposes of quantitative MID, an internal standard of the PGE₂ derivative was prepared with deuterium-labeled d_6 -acetic anhydride; the d_6 -labeled derivative (bis d_3 -acetate) fragments by losing the elements of d_3 -acetic acid (63 a.m.u.), but the major ion at m/e 422 (base peak) still retains one deuterium-labeled moiety. Figure 3 is a reproduction of the actual MID data trace resulting from a series of injections of prostaglandins at physiologically significant levels. Relatively large quantities (70-fold excess) of the deuterium-labeled carriers were co-injected to minimize adsorptive losses of the endogenous (or unlabeled) species. In Figure 3, the peak on the left (at the X100 level) was derived from 32 ng of PGE₂-MO-ME-bis- d_6 -bis acetate (carrier) and 480 pg of PGE₂-MO-ME-bis acetate; the major portion of the peak was generated by m/e 422 from the former (labeled, carrier) compound, and the small "interior" portion of the peak was produced by m/e 419 from the latter (unlabeled) compound. The middle peak in the data tracing was derived from 32 ng of labeled, carrier PGE₂-MO-ME-bis- d_3 -acetate only. Note that the contribution of m/e 422 to the peak is essentially the same as in the previous peak, but the contribution of the carrier compound to m/e 419 or the interior portion of the peak on the right is almost negligible. However, the fact that deuterium-labeled carrier does contribute slightly to m/e 419 is one of the factors limiting the useful detection

level of this method. The area generated by injection of 480 pg plus carrier ("interior" of peak on left) is twice that resulting from injection of the carrier alone ("interior" of middle peak).

A small laboratory computer (PDP-12) has been interfaced with the GC-MS (LKB-9000) to control the operation of the MID hardware as well as perform on-line acquisition and reduction of the data (9). Use of the conventional oscillograph often produces a confusing record of MID data because the recording medium (a light beam) is not interrupted as the accelerating voltage is switched alternately between preset values. As a result, the oscillographic MID record is essentially a series of transient sweeps, the end points of which indicate the abundance profiles of the selected ions. The results of conventional oscillographic data recording and oscilloscopic presentation (from the computer) are compared in Figure 4. These records result from approximately 10 ng of a mixture of labeled and unlabeled prostaglandin E as the methyl ester methoxime, bis-acetate derivative. Ions of m/e 388 and 419 are produced by the unlabeled species and m/e 422 is derived from the labeled molecule. The upper left panel of Figure 4 is the conventional oscillographic trace in which the outline of all three profiles should be evident. The same data were also acquired by the computer and are represented in the upper right panel of Figure 4 as a reversed photograph of the CRT (oscilloscope) screen on the computer. Although the three profiles on the CRT are accompanied by mass numbers in the upper right, the question still remains: "Which profile is which?" The operator can easily distinguish the different profiles, however, by manipulating switches on the computer console to remove individual profiles with its corresponding m/e value from the CRT. This process is illustrated in the lower panels of Figure 4 in which the ion profile of m/e 422 and then that of m/e 388 are removed from the CRT display.

The reduced MID data are stored by the computer in a format readily available for computation of peak areas. By using one of the ten subroutine options in the MID software, the operator can display on the CRT any selected ion current profile along with three adjustable cursors (which resemble three sides of a rectangle as displayed on the CRT) which can be adjusted to define limits of the peak area to be integrated. After adjusting the area-limiting cursors to the leading and trailing edges and the baseline of the peak, the operator presses the "C" key on the teletype console; the computer then calculates the area and prints a numerical value on a teletypewriter. The acquisition, reduction, and data handling by computer reduces human error and should lead to better precision in low-level quantification.

ACKNOWLEDGMENT

The encouragement of work by Dr. John A. Oates and support from the Center for Clinical Pharmacology and Drug Toxicology, NIH-CN-15431 are gratefully acknowledged. Samples of the prostaglandins were a gift from Dr. J. Pike of the Upjohn Company.

References

1. Watson, J. T., Vanderbilt University, Nashville, Tenn., Unpublished results.
2. Sweeley, C. C., Elliott, W. H., Fries, I., and Ryhage, R. *Anal. Chem.*, 38, 1549 (1966).
3. Hammar, C.-G., Holmstedt, B., and Ryhage, R. *Anal. Biochem.*, 25, 532, (1968).
4. Samuelsson, B., Hamberg, M., and Sweeley, C. C. *Anal. Biochem.* 38 301, (1970).
5. Hammar, C.-G. and Hessling, R. *Anal. Chem.*, 43, 298, (1971).
6. Gaffney, T. E., Hammar, C.-G., Holmstedt, B., and McMahon, R. E. *Anal. Chem.*, 43, 307, (1971).
7. Klein, P. D., Haumann, J. R., and Eisler, W. J. *Anal. Chem.*, 44 490, (1972).
8. Sweetman, B. J., Frolich, J. C., and Watson, J. T. Department of Pharmacology, Vanderbilt University, Unpublished results.
9. Pelster, D., and Watson, J. T., Department of Pharmacology, Vanderbilt University, Unpublished results which were presented initially at the 5th Great Lakes Regional ACS Meeting, Peoria, Illinois, June 11, 1971.

Figure 1. Parallel gas chromatograms. Upper panel: Total ion current profile from GC-MS; lower panel: MID oscillographic record of same sample.

Figure 2. Abbreviated mass spectrum of normetanepherine as the pentafluoropropionyl derivative.

Figure 3. Reproduction of actual MID oscillographic record resulting from three separate injections of indicated amounts of unlabeled (m/e 419) and labeled (m/e 422) Prostaglandin E₂ as the methylester, methoxine, bis-acetate derivative.

Figure 4. Comparison of oscillographic (upper left) and oscilloscopic presentation (from PDP-12 computer) of MID data of m/e 388, 419, and 422. The upper panels are parallel recordings of the composite group of ion profiles; the lower panels illustrate a sequence in which the ion profile of m/e 422 and then that of m/e 388 are removed from the oscilloscope screen.

DISCUSSION

Dr. Dinerstein: I would like to direct a remark to Dr. Watson. You stated your goal or direction is to develop a simple direct method for analyzing blood samples using a simple system, perhaps electron capture gas chromatography. My impression is that the beauty of the MID is a nice flat baseline. The elimination of the problem of column contamination is a significant improvement in itself.

Dr. Watson: I see what you are getting at. One does enjoy, with multiple ion detection, a technique that has a very high, and in fact adjustable, specificity so that he can adjust it to meet his compound of interest. You have to be careful with the system I am using, that the ions you want to monitor fall within 10% of one another. Dr. Klein's new development has a spread of $\pm 50\%$, a much broader range. But all of us have to make sure that the ions of interest don't lie in a region to which the background from the column contributes. This will surprise you because it doesn't look very big on a normal scan. We usually open the slits and this makes a giant peak out of an otherwise insignificant background. In fact, one can sit and monitor a fair portion of the spectrum in this manner. This is all a function of the stationary phase one uses.

Dr. Dinerstein: This is a trivial point, but what is the difference between administering the labeled compound and making a labeled derivative of the compound as you did?

Dr. Watson: We had to do that because most of the things we would like to talk about are not available as deuterium-labeled or carbon-13 labeled analogs. It is advantageous to have these because one can, for example, add the internal standard directly to the plasma sample or urine sample and then that internal standard is effective all the way through the methodology. If one installs the label in the derivative one still encounters a variety of methodological losses for which one usually needs radioactive means of monitoring.

Dr. Wall: Is the internal standard administered to the animal, or do you add it before extracting the plasma?

Dr. Watson: In our cases it is after extracting it from the animal. This bit about the toxicity of deuterium is not a problem if one is interested in quantification of endogenous material when one adds the label to the sample after extraction.

Another point is the price comparison between gas chromatography and electron capture technique--what we are doing is very expensive. On the other hand, Dr. Klein's point is that if a bunch of us show there is a need for this MID technique, perhaps it can be manufactured less expensively.

USE OF STABLE ISOTOPES IN PHARMACOLOGY - CLINICAL PHARMACOLOGY

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Advantages and Disadvantages of Stable Isotopes

The greatest advantage offered by stable isotopes over their radioactive counterparts is the absence of problems associated with radiation. The lack of possible radiation damage to living systems is very important in human studies and especially where clinical biochemistry or pharmacology is being done in children or pregnant women. This lack of radiation hazard makes the synthesis and handling of stable isotope-labeled compounds much simpler. The stability of the compounds is less of a problem since there is no decay or radiolytic decomposition to contend with. In some cases, most notably that of deuterium versus tritium, the smaller mass difference of the stable isotope in relation to the most abundant natural isotope makes for a smaller kinetic isotope effect, a source of confusion in certain experiments. In the case of nitrogen and oxygen, the stable isotopes, ^{14}N and ^{16}O or ^{17}O are without competition since there are no radioactive isotopes of sufficiently long half-life to be useful as labels. Stable isotopes are not without their disadvantages however. The greatest single disadvantage with stable isotopes is the lack of a simple, sensitive, inexpensive isotope specific method of quantitation comparable to liquid scintillation counting. With radioactive labels one has a very convenient inexpensive and sensitive non-compound specific means of detection--namely that of detecting the emitted radiation. Modern liquid scintillation counters can easily detect labeled drugs in picogram amounts, assuming typical specific activities in the 1-100 millicurie per millimole range. Radiation detectors lack specificity, however, since the amount of isotope present in the sample is measured without regard to its chemical or molecular form. In contrast, the mass spectrometric technique of mass fragmentography (1) provides a molecule-specific detection method whose sensitivity rivals that of radioisotopes in favorable circumstances, e.g., chlorpromazine has been detected in picogram amounts (1). Unfortunately, this latter technique requires more expensive instrumentation and is associated with greater analytical complexity than detection techniques such as liquid scintillation counting.

Uses of stable isotopes in pharmacology are primarily as tracer labels for drug distribution and metabolism studies. The first use of stable isotope tracers in a biological study was reported in 1934 (2). The same general technique of administering a labeled compound and determining the stable isotope abundance in an isolated product is, with refinements, still in use today (3). These isotopes may also be useful to the pharmacologist or biochemist as internal standards for quantitative measurements with mass spectrometry or gas chromatography-mass spectrometry.

Stable Isotope Mixtures as Tracers for the Identification of Drugs and Drug Metabolites by Simple Mass Specific Scanning

Although mass spectrometry is the principle method used for analysis of compounds labeled with stable isotopes, there is nothing diagnostic about the mass spectrum of the stable isotope-labeled compound except for the shift due to the increased mass. Unless the mass spectrum of the unlabeled compound is known a labeled compound cannot be recognized *a priori*. Thus there is no way, simply by mass spectral analysis, to distinguish the stable-labeled compound from other molecular structures which are present in the complex mixtures obtained from biological material. This difficulty can be overcome however by the use, or administration, of mixtures of stable isotope-labeled and unlabeled compounds. Such mixtures can be used to artificially create a visually conspicuous isotopic cluster in the mass spectrum. Such doublets can serve as an easy means of identifying stable-labeled compounds be they parent drug or drug metabolites.

Krapp *et al.* (4) have called attention to this application using m , $m+3$ doublets created by trideuterium-labeled and dideuterium- ^{15}N -labeled drug (Fig. 1). Vore *et al.* (5) have reported on the same use of an m , $m+1$ doublet created with an ^{15}N label in a barbiturate metabolism study. The m , $m+2$ and m , $m+3$ doublets are preferable to m , $m+1$ doublets since natural isotopic contributions can contribute significantly to $m+1$ peaks. This technique makes use of the mass spectrometer, not only to identify the labeled chromatographic peaks in a complex chemical mixture but also exploits the ability of the instrument to give information on the structure of the compounds and the location of the label.

The need for such an isotope cluster label has been questioned; the point being made that an experienced mass spectroscopist can identify metabolites without this gimmick. The technique can, however, be of much help to the less experienced worker and can also be a big time-saver for the mass spectroscopist in that it allows him to concentrate his attention only upon those peaks in a complex chromatogram which carry the label.

Stable Isotope Labels as an Aid to Ion-Specific Detection of Drugs and Their Metabolites by Mass Fragmentography

By using a multiple ion detector accessory with a gas chromatograph-mass spectrometer, the mass spectrometer can be made to serve as a very sensitive and molecule specific gas chromatographic detector. As little as one picogram of a single molecular species has been seen with this technique (1). The technique, sometimes called mass fragmentography (1), involves continuous monitoring of several characteristic ion fragments of the molecule under study; preselected mass numbers are recorded instead of scanning the complete mass spectrum. In this way quantities of compounds, insufficient to provide a characteristic mass spectrum, can easily be detected in a highly specific manner. The mass spectrometer "detector" then only "sees" compounds giving fragments at the selected mass numbers. Molecular identity is confirmed by the characteristic ion intensity ratio(s) and the gas chromatographic retention time. For maximum sensitivity with this technique the most intense peak in the mass spectrum of the compound is monitored, i.e., the base peak. Unfortunately, the mass range within which one can continuously monitor several ions is limited by the instrumental technique. If a second intense fragment ion does not lie within the narrow accessible mass range adjacent to the base peak, the use of ion-intensity ratios as a criteria of molecular identity is precluded or much reduced.

The usefulness of the mass fragmentography technique would be optimized if 1) each molecule under study had a second fragment ion of equal intensity to the base peak and 2) if the second ion was separated from the base peak by more than one mass unit to avoid interference from natural isotopes but less than about 4 atomic mass units to facilitate easy and accurate focussing. This ideal ion combination can be artificially created in a drug and its metabolites as mentioned earlier by the administration of an equal molar mixture of unlabeled drug and drug suitably labeled with stable isotope(s). This one-to-one intensity ratio of the two specific mass numbers provides a high degree of molecular specificity in addition to the sensitivity of the mass fragmentography technique.

Knapp *et al.* (6) have evaluated the usefulness of this artificially-created cluster technique in the detection of trace quantities of drug metabolites in human urine (Fig. 2) and bile (Fig. 3). The technique has also been used to detect small amounts of drug metabolite formed in isolated perfused organs (Fig. 4) (7). These examples illustrate how the specificity enhancement of the mass fragmentography technique using stable isotopes is especially useful when searching for very small quantities of a compound in a complex biological mixture. Single-ion monitoring often yields a multitude of peaks when chromatographing such a mixture. An extraneous peak eluting near where a compound of interest is expected is easily misinterpreted without the added specificity of a peak-ratio measurement.

Since electron capture detection has comparable sensitivity to mass fragmentography one might question whether the latter is worth the additional expense and effort. If the necessary derivatives can be made and if the ECD technique can be shown to have adequate selectivity it would be wiser to use it. In fact, the stable isotope label-mass fragmentography technique can be used to confirm the specificity of an ECD method. However, in those cases where suitable ECD derivatizations are not available, e.g., tertiary amines, and where ECD interference is present in a complex mixture, or where the specificity of existent GC-ECD methods have not been confirmed, the isotope cluster-mass fragmentography method fills a definite need.

This need for new highly sensitive and selective analytical techniques is an important one in pharmacology. Highly potent drugs must necessarily be administered in small doses resulting in very small amounts of drug and metabolites present for analysis. Even less potent drugs are often distributed so widely that the amount present in a given tissue sample might be extremely small. Size limitations of certain samples (e.g., needle-biopsy specimens and finger-stick blood samples) also require very sensitive analytical methods. More sensitive analytical methods are needed for investigation of minor pathways of drug metabolism. The observation that less than 10% of methyl dopa is converted to metabolites which are responsible for the hypotensive effects of the drug emphasizes the need to be alert to the significance of small metabolic pathways of drugs (8). The availability of highly sensitive and selective analytical techniques has important implications in the study of new drugs in man. With prior knowledge of the metabolism of a drug in lower species, suspected metabolites can be sought in man using very low dosages. This allows qualitative metabolism studies to be carried out with much smaller and therefore safer doses, an important consideration since this work is done at a time when little is known about the effects of the drug in man.

When using stable isotope labels to create mass spectral clusters, possible intensity distortions due to kinetic isotope effects in the metabolic reactions must be kept in mind. For example, a significant deuterium isotope effect has been demonstrated in the dealkylation of amphetamine-type amines in man (Fig. 5) (9). Such considerations must enter into the choice of labeling sites.

Stable Isotopes as an Aid to Distinguish Drug Metabolites from Natural Products

Another use of stable isotope labels in pharmacology is the case where an administered drug is thought to be converted to a metabolite which is also present as a natural product. Without a label one can only demonstrate a change in the concentration of metabolite or natural product. With appropriate labeling with a stable isotope one can demonstrate that the metabolite came from the administered drug and that the change in the level of compound in question was not simply a secondary effect. A specific example will illustrate. *m*-Naphthol has recently been observed by Walle *et al.* to be a metabolite of

propranolol (10). Unfortunately, *p*-naphthol also occurs in the urine of control subjects. To distinguish *p*-naphthol derived from propranolol from other sources of *p*-naphthol the drug could be ring labeled with deuterium. Proof could also be attempted using a radioisotope, however this approach would require separate isolation and purification of the *p*-naphthol. A stable label in conjunction with GC-MS is simpler, does not require an additional isolation step and provides a molecule-specific criterion of identity, i.e., the mass spectrum of the compound under question shifted by the presence of the stable isotope.

Stable Isotope Labels in Conjunction with Radioisotopes

Stable isotope mixtures may also be used with compounds containing a radioactive tracer. The radioactive isotope tracer allows highly sensitive radiation specific detection for ease of isolation and quantitative measurement; the stable label can be seen in the complete mass spectrum if the concentration allows or in the mass fragmentogram if only trace quantities of the drug or its metabolites are present. This combination of stable and radioactive labeling provide the advantages of both types of isotopes. Of course one also retains the problems attendant to handling radioisotopes. Such double labeling was first used in a study of methionine transmethylation using ^{14}C and deuterium labeling on the methionine methyl (11). Vore (3) has combined both types of labeling in the barbiturate containing a 50% ^{15}N labeled site as well as a ^{14}C labeled site. The importance of having both labels on the same molecule, i.e., intermolecular versus intramolecular, has been pointed out since kinetic isotope effects can cause problems with intramolecular labeling (12). By using the combination of ^{13}C , ^{14}C or deuterium, tritium it would be possible to put both labels at the same site. This combination of stable and radioactive isotopes in conjunction with the GC-MS containing a radioactivity detector (13) on the gas chromatograph would appear to be a particularly powerful tool.

Stable Isotope-Labeled Compounds as Internal Standards for Quantitative GC-MS Involving Drugs and Natural Products

The isotope dilution method for quantitative determination was introduced by Rittenberg (14) in 1940 using stable isotopes. He used deuterium-labeled fatty acids and ^{15}N labeled amino acids as adducts in quantitative determinations of these compounds. The subsequently isolated, purified and combusted compounds were then examined for their relative isotopic abundance using an isotope ratio mass spectrometer. This is the same principle which underlies the determination of specific activity in the radioactive isotope dilution method.

The availability of the combined gas chromatograph-mass spectrometer instrument has made this technique applicable to intact molecules, i.e., has eliminated the necessity of the combustion step and the need for separate isolation and purification steps. Samuelsson *et al.* (15) (Fig. 6) have suggested this technique for the quantitative analysis of a natural product prostaglandin E_1 . Gaffney *et al.* (16) have suggested the technique for the quantitative measurement of the

drug nortriptyline in human plasma (Fig. 7). In these techniques the stable isotope labeled internal standard was added to the crude sample which was subjected to gas chromatographic separation and mass spectrometric analysis. In the drug application, the labeled drug was added prior to extraction, derivatization, and analysis. In the prostaglandin application the internal standard was added as deuterated methoxime derivative, a less desirable approach since prior extraction losses and variation in derivative formation from the natural material could introduce errors. In both these applications multiple ion detection (17) was used to determine the ratio of labeled to unlabeled compounds. In this technique the m/e peak intensity must be integrated over the entire chromatographic peak since the labeled and unlabeled compounds cannot be assumed to have identical retention times (Fig. 8) (18).

The discussion of the relative merits of the isotope cluster-mass fragmentography technique versus ECD are relevant here also. If a simpler technique will do the job, it is to be preferred, but in cases where ECD fails (tertiary amines for example) the additional expense and effort of the stable isotope internal standard technique is justifiable.

ACKNOWLEDGMENT

The authors would like to acknowledge the interest, enthusiasm, and intellectual support of Dr. C.-G. Hammar, Kabi Research Group, and Dr. R. E. McMahon, Lilly Research Laboratories, for the pharmacological uses of stable isotopes.

The library and secretarial skills of Mrs. Fran Donlan were of great help in the preparation of this manuscript.

REFERENCES

1. Hammar, C.-G., and Holmsted, B. Mass Fragmentography: Identification of Chlorpromazine and Its Metabolites in Human Blood by a New Method, *Anal. Biochem.* 25, 532-548 (1968).
2. Havesy, G., and Hofer, E. How Long Does Water Remain in the Body ("Heavy" water as indicator), *Klin. Wochschr.* 13, 1524 (1934); cited from D. Rittenberg, *The Influence of Nuclear Mass on Biological Systems, in Isotopic and Cosmic Chemistry*, ed., by H. Craig, S. L. Miller, and G. J. Wasserburg, North-Holland, Amsterdam, 1964, p. 60.
3. Fürst, P., and Jonsson, A. Control and Modification of Methods for Determination of ^{15}N in Biological Material, *Acta Chem. Scand.* 25, 930-938 (1971).
4. Knapp, D. R., Gaffney, T. E. and McMahon, R. E. Use of Stable Isotope Mixtures as a Labeling Technique in Drug Metabolism Studies *Biochem. Pharmacol.*, 21, 425-429 (1972).
5. Vore, M., Gerber, N., and Bush, M. T. The Metabolic Fate of 1-n-butyl-5,5-diethylbarbituric Acid in the Rat, *Pharmacologist*, 13, 220 (1971).
6. Knapp, D. R., Gaffney, T. E., McMahon, R. E., and Kiplinger, G. Studies of Human Urinary and Biliary Metabolites of Nortriptyline Using Stable Isotope Labeling, *J. Pharmacol. Exptl. Therap.*, 180 784-790 (1972).
7. Whitnack, E., Knapp, D., Fowler, N., Holmes, J., and Gaffney, T. E. Metabolism of Nortriptyline in the Lung, *J. Pharmacol. Exptl. Therap.* 181, 288-291 (1972).
8. Buhs, R. P., Beck, J. L., Speth, O. C., Smith, J. L., Trenner, N. R., Cannon, P. J., and Laragh, J. H. The Metabolism of Methyldopa in Hypertensive Human Subjects, *J. Pharmacol. Exptl. Therap.* 143, 205 (1964).
9. Vree, T. B., Gorgels, J. P. M. C., Muskens, A. Th. J. M., and Van Rossum, J. M. Deuterium Isotope Effects in the Metabolism of N-Alkyl-substituted Amphetamines in Man, *Clin. Chim. Acta* 34, 333-344 (1971).
10. Walle, T., and Gaffney, T. E. Metabolism of Propranolol in Man: Mass Spectrometric Identification of Four New Metabolites, *Pharmacologist*, 13, 221 (1971).
11. Keller, E. B., Rachele, J. R., and duVigneaud, V. A Study of Transmethylation with Methionine Containing Deuterium and C^{14} in the Methyl Group, *J. Biol. Chem.* 177, 733-738 (1949).

12. Rachele, J. R., Kuchinskas, E. J., Knoll, J. E., and Eidinoff, M. L. Hydrogen Isotope Effects in the *In Vivo* Utilization of Formate, *Arch. Biochem. Biophys.*, 81, 55-62 (1959).
13. Hobbs, D. C. Eighteenth Annual Conference on Mass Spectrometry and Allied Topics, June 14-19, 1970, San Francisco, California.
14. Rittenberg, D., and Foster, G. L. A New Procedure for Quantitative Analysis by Isotope Dilution with Application to the Determination of Amino Acids and Fatty Acids, *J. Biol. Chem.*, 133, 737-744 (1940).
15. Samuelsson, B., Hamberg, M., and Sweeley, C. C. Quantitative Gas Chromatography of Prostaglandin E₁ at the Nanogram Level. *Anal. Biochem.*, 38, 301-304 (1970).
16. Gaffney, T. E., Hammar, C.-G., Holmstedt, B., and McMahon, R. E. Ion Specific Detection of Internal Standards Labeled with Stable Isotopes, *Anal. Chem.* 43, 307-310 (1971).
17. Sweeley, C. C., Elliot, W. H., Fries, I., and Ryhage, R. Mass Spectrometric Determination of Unresolved Components in Gas Chromatographic Effluents, *Anal. Chem.*, 38, 1549-1553 (1966).
18. Klein, P. D. The Occurance and Significance of Isotope Fractionation During Analytical Separations of Large Molecules. In *Advances in Chromatography*, Vol. 3, ed. J. C. Giddings and R. A. Keller, Marcel Dekker. New York, 1966, pp. 3-64.

Figure 1. (Top) Total ion current recording from gas chromatographic separation of a basic urinary extract from a nontriptyline isotope mixture dosed rat.

(Bottom) Partial mass spectrum from scan 10 showing m/e 357, 360 m, m+3 doublet resulting from the mixture of stable isotope labeled and unlabeled compound. (from reference 4)

Figure 2. Total ion current recording (A) and mass fragmentograms (B,C) of nortriptyline post-medication basic urine extract and reference compound mixture (D). Compounds in B have m, m+2 base peak doublet (from reference 6).

Figure 3. Mass fragmentogram of nortriptyline postmedication basic bile extract. Broken line is total ion current recording. Parent drug and metabolites have m , $m+2$ doublet in base peak (from reference 6).

Figure 4. Identification of desmethylnortriptyline metabolite in dog lung tissue extract (A) by mass fragmentography on the m/e 232, 234 base peak doublet. Mass fragmentogram of extract from isolated heart preparation (B) showing no desmethyl metabolite (from reference 7).

Figure 5. Renal excretion rates of isopropylamphetamine and deuterioisopropylamphetamine showing large isotope effect beginning about 10 hours after ingestion (adapted from reference 9).

Figure 6. Ratios of measured peak areas of the protium form and d_3 -form of derivatized prostaglandin E_1 versus the composition of the injected material showing linearity over the range of 1-8 nanograms of protium form (from reference 15).

Figure 7. Base peak mass fragmentograms of 50 picograms each of TFA-nortriptyline (m/e 232) and TFA- d_2 -nortriptyline (m/e 234) (from reference 16).

Figure 8. Partial gas chromatographic resolution of a silylated mixture of glucose and d_7 -glucose observed by multiple ion detection of three pairs of fragment ions (from reference 17).

CODING OF BARBITURATE SOURCES BY STABLE ISOTOPE LABELING
DURING MANUFACTURE*

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According to the United State Tariff Commission (1), the U. S. production of barbiturates in 1968 was 770,000 lbs, which if calculated on the basis of a 100 mg capsule represents some 3.5×10^9 doses, or enough to provide an average of 17 doses for every man, woman, and child in the U. S. Moreover, according to the former director of the division of narcotic addiction and drug abuse of the National Institute of Mental Health, Dr. Sidney Cohen, at least half of this supply enters the illicit market (2).

We became interested in the feasibility of tracing the point of diversion from legitimate channels by incorporating a stable isotopic code in all barbiturates at the time of manufacture. Such a code, when read in confiscated samples, could indicate not only the manufacturer, but lot number or period of production as well. This would serve to narrow the number of diversion possibilities by subdividing the production into sequential time periods, during which shipments to different distribution points could be distinguished.

There are several factors which suggest that such a coding process could be successfully employed. The first is the limited number of manufacturers who supply the bulk barbiturates to the pharmaceutical companies who in turn incorporate them into their own formulations. While there are literally hundreds of formulations, Table 1 shows that there are only six basic producers and any given type of barbiturate is likely to have no more than three possible sources.

* Work supported by the U. S. Atomic Energy Commission.

** Work conducted under the Central States Universities, Inc. program. Present address: Dept. of Chemistry, University of California, Los Angeles.

TABLE I

Barbiturate Manufacturing Sources (1)

Barbiturate Product	GAN*	SDW	ABB	LIL	PD	MAL
Allylbarbituric acid	x					
Amobarbital				x		
Barbital	x					
Butabarbital	x		x			
Cyclobarbital		x				
Hexobarbital	x	x				
Mephobarbital		x				
Metharbital			x			
Methohexital				x		
Pentobarbital	x		x			
Phenobarbital	x	x				x
Secobarbital	x			x		
Talbutal					x	
Thiamylol			x			
Thiopental		x				
Totals: 15	7	5	4	3	1	1

*

GAN: Ganes Chemical Works, Inc.

SDW: Sterling Drugs, Inc., Winthrop Laboratories Div.

ABB: Abbott Laboratories

LIL: Eli Lilly & Co.

PD: Parke, Davis and Co.

MAL: Mallinckrodt Chemical Works.

The second factor is that all forms of barbiturates involve the same synthetic process; more specifically, all involve the condensation of a malonic acid ester with urea. This intermediate which is common to all products offers the possibility of introducing a three digit, non-adulterable identification code into all products by requiring all manufacturers to include a Federally-supplied labeled urea in their feed stock. Such a urea can be labeled in any combination of $1\text{-}^{15}\text{N}$, $1,3\text{-}^{15}\text{N}$ or $1,3\text{-}^{15}\text{N}\text{-}2\text{-}^{13}\text{C}$ to give three distinctive enrichments at $m+1$, $m+2$ and $m+3$. The possibility of three distinctive label combinations in such a small molecule is particularly attractive from an economic standpoint.

The third factor favoring successful coding is the universal characteristic of all barbiturate mass spectra, as shown in Figure 1 for pentobarbital, to exhibit a large, invariably base, peak representing the intact ring structure. This ion is always a product of the fragmentation processes, though its mass number may vary slightly according to the nature of alkyl substituents. Most importantly, it always contains the three labeling atoms. Thus the code can be read by the same means for any variety of barbiturate molecule.

The fourth factor is that the sensitivity of labeling increases enormously with multiple labeling, yet does not require a corresponding increase in the absolute instrument sensitivity. Table 2 lists the natural isotopic contributions to the $m+1$, $m+2$ and $m+3$ ions for the base peak of m/e 169, as well as their relative intensities. By measuring the ratio between successive peaks, e.g., 170/169, 171/170 and 172/171 instead of the ratio to the base peak, the dynamic range of the ratio is kept narrow and well within the present instrumental capabilities.

TABLE II

Natural Isotopic Abundances and Relative Intensities for
N,N' Dimethyl Pentobarbital at Various Masses

m/e	Natural Isotopic Abundance	Relative Intensity
169	89.974	100.0
170	8.730	9.703% of 169
171	1.209	1.344% of 169 13.85% of 170
172	0.087	0.097% of 169 0.10% of 170 7.2% of 171

Accordingly, singly and doubly labeled pentobarbitals were synthesized from $1-^{15}\text{N}$ and $1,3-^{15}\text{N}$ -labeled urea by the method of Murray and Ronzio (3). They were derivatized for gas chromatography-mass spectroscopy by conversion to the 1,3 dimethyl derivatives (4) and chromatographed on a 3% SE-30 column. Dilution curves of the isotopic materials were obtained with the Argonne GMA (gas chromatograph-mass spectroscopy-accelerating voltage alternation) system previously described (5). These curves are shown in Figures 2 and 3. They show that the singly labeled material can be measured at a dilution of 0.1% and the doubly labeled material at 0.02%. Based upon the natural isotopic abundance at m/e 172 and the instrument capabilities demonstrated here, we anticipate that, when available, triply labeled urea would permit measurement of labeled pentobarbital at dilutions as low as 0.001% (Figure 4).

The economic feasibility of such a code hinges on three factors: The first is the cost of labeled urea used in coding, and in particular the magnitude of such costs with regard to the overall cost of barbiturate manufacture. This is directly related to the second factor, namely the cost of stable isotopes used to prepare the coded urea. The growth in production under the Los Alamos ICONS program and the attendant decline in cost of ^{13}C and ^{15}N suggest that using present

costs as a guideline would be highly conservative. Moreover, the cost is related to a third factor; the extent to which the isotope can be diluted and still preserve a sufficient number of discreet, distinguishable enrichments, for the lower the detectable concentration, the less isotope that is required.

As a preliminary estimate of the economic factors, consider the production figure cited earlier of 770,000 lbs of barbiturates. Neglecting for the moment the efficiency of the synthesis procedure, the final product represents the incorporation of 220,000 lbs of urea. The value of bulk barbiturates as a chemical commodity in 1968 was estimated by the U.S. Tariff Commission to be \$4.93 per pound (1). If, for the sake of argument, we stipulate that such labeling might be feasible only if the added cost did not exceed 10% of the bulk price, labeling costs of \$0.49 per pound would be permissible. This corresponds to \$1.70 per pound, or 3.76¢ per gram of urea used in the manufacture. If the present cost of triply-labeled urea were taken to be \$376 per gram, (and in small lots this is the approximate market cost) the average enrichment level in the code would have to be maintained at 0.01% or lower. This would be achieved by a total of 22 lbs of urea. If, on a larger scale of production, the cost of labeled urea were reduced to \$37.60 per gram, a ten-fold increase in the weight of urea to 220 lbs would permit labeling at levels as high as 0.1% and would provide an enormous number of coding levels and combinations.

It appears therefore that, given the increase in ^{13}C and ^{15}N production promised by the ICONS program and a manifest interest on the part of the drug manufacturers, the Food and Drug Administration, and the Division of Narcotics and Dangerous Drugs, such coding and identification has economic and analytical practicality.

REFERENCES

1. "United States Production and Sales of Medicinal Chemicals, 1968" United States Tariff Commission, Jan. 1970, p. 17.
2. Chemical and Engineering News, Nov. 2, 1970, p. 26.
3. Murray, A. III, and Ronzio, A. R. in "Organic Syntheses with Isotopes" A. Murray, III and D. L. Williams, eds. Interscience Publishers, Inc., New York, 1958, Part 1, p. 711.
4. Stewart, J. T., Duke, G. B. and Willcox, J. E. *Analyt. Letters*, 2, 499 (1969).
5. Klein, P. D., Haumann, J. R., and Eisler, W. J. *Anal. Chem.* 44, 490 (1972).

Fig. 1. Mass spectrum of N,N'-dimethylpentobarbital

Fig. 2. Experimental dilution curve for singly labeled 1-¹⁵N-pentobarbital

Fig. 3. Experimental dilution curve for doubly labeled (1,3-¹⁵N)-pentobarbital

Fig. 4. Relative intensities and dilution limits for pentobarbital labeled in one, two, or three atoms.

AN ANALOG MULTIPLEXER FOR MULTIPLE SPECIFIC ION DETECTION
IN A GAS CHROMATOGRAPH/QUADRUPOLE MASS SPECTROMETER SYSTEM

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ABSTRACT

A programming device has been constructed which switches the mass filter of QUAD 300 quadrupole mass spectrometer sequentially through up to eight m/e values, and synchronously switches the electrometer output through a corresponding set of output channels. The outputs are smoothed and may either be recorded on a multichannel recorder or combined linearly to provide data channels which are specific for isotopic variants of a single gas chromatographic peak. The m/e values may be selected in any order over the whole mass range of the instrument.

Sweeley *et al* (1) first applied the principle of alternate focussing of a mass spectrometer on two different m/e values to achieve the resolution of two components of a gas chromatographic peak and to measure the isotope ratio in a single peak. This technique has since been widely used for the identification of peaks eluting from a gas chromatograph (2), and for accurate quantitation of components through the use of stable isotope-labeled carrier which also serves as an internal standard (3,4). These studies have employed a magnetic instrument, the focus of which is switched by changing the accelerating voltage. The mass filter of a quadrupole mass spectrometer may be switched with greater facility and over a greater mass range (5), and a peak programmer with up to eight channels is available commercially as an option on an EAI (Electronic Associates Incorporated, 4151 Middlefield Road, Palo Alto, California 94303) QUAD GC/MS system. Nevertheless, this has received little attention in pharmacological investigations. In this report a simple, stable and highly reliable device is described, which employs integrated circuit FET switches to multiplex and demultiplex the program voltage and electrometer output of the QUAD 300 respectively. Eight individual outputs are provided, each of which gives a smoothed record of the ion current at a specific m/e value as a function of time.

Figure 1 summarizes the operation of this device. A multivibrator operating at about 100 Hz serves as a clock which drives a 3-bit counter. Through decoder and driver stages, two sets of analog switches (MM554F) are synchronously driven. One set sequentially switches eight preset voltages into the program input of the QUAD 300; these voltages are set by potentiometers on a precision voltage reference to correspond to the required m/e values. The second set of analog switches sequentially directs the electrometer output to a series of eight outputs, so that each of the eight outputs corresponds to a pre-determined m/e value. In operation each of the outputs consists of a rectangular pulse lasting approximately 10 ms with an amplitude equal to the electrometer output, at a repetition rate of 12 pps. They are averaged by four-stage RC filters with time constants of 0.5 sec. In this way a spectrum may be obtained of any compound eluting from the column at up to eight m/e values, which is sufficient for positive identification of an unknown when a reference spectrum is available. Crosstalk between channels is not detectable provided that a sufficiently fast electrometer is used to measure the electron multiplier current, and is less than -80 dB when the electrometer time constant is 10 ms. Each output is equipped with a set of separate gain controls consisting of operational amplifiers with variable negative feedback. They can either be used to drive different channels of a multichannel potentiometric recorder, or can be fed into summing circuits to form linear combinations of up to four m/e outputs and a constant. The constant allows a bucking potential to be applied to compensate for column bleed or electrometer offset. One of the coefficients of the four m/e outputs may be chosen to subtract the solvent tail to provide a flat baseline for peak measurement when very small quantities are being measured; the remaining three may be chosen so that when more than one isotopic variant of the same component is being analyzed, the outputs of each summing circuit will be proportional to the quantities of each isotopic variant and are therefore component-specific rather than mass-specific. This is not, in general, true of the outputs corresponding to individual m/e values since each isotopic variant will contribute to some extent to the output at each m/e value; the coefficients needed to obtain component-specific operation can be calculated by inverting the matrix of mass spectra for the three components at the three m/e values recorded.

For many applications this system offers several advantages over the accelerating voltage alternator available as an accessory to the LKB-9000 and the multiple-ion-detector described by Hammar and Hessling (6). It allows the simultaneous display of the outputs from up to eight m/e values, as independent variables without switching artifacts; the m/e values are not restricted to any region of the spectrum but may be selected at random from any part of the mass range; the system is inherently more stable because it does not contain a drift-prone magnetic component and because the low (unit) resolution makes the setting of the mass filter less sensitive to small changes in sensitivity to program voltage; and the total cost of the system is considerably less because

of the simple and compact design permitted by modern integrated circuits. The quadrupole system does not, of course, permit precision mass measurements by peak matching as Hammar and Hessling's device does (6), but it has proved highly satisfactory for quantitative GC/MS analyses in which stable isotope labeled internal standards and tracers are used. The outputs may readily be interfaced with an analog to digital converter and digital data processing equipment for integration of gas chromatographic peaks and automatic analysis. The system meets most of the design criteria recently set out by Klein *et al* (7) for the clinical application of stable isotope analysis.

It is now being used for studies of acetylcholine turnover in which $^2\text{H}_9$ -labeled choline (I) and acetylcholine are used as internal standards and $^2\text{H}_4$ -labeled choline (II) is used as a labeled precursor.

Using the demethylation procedure previously described for the gas chromatographic analysis of quaternary ammonium compounds (8,9), it is possible to estimate simultaneously the endogenous, $^2\text{H}_4$ - and $^2\text{H}_9$ -labeled choline and acetylcholine by programming a QUAD 300 mass spectrometer at m/e 58, 60, and 64, the base peaks of the three isotopic variants after derivatization. The detection limit in this application is approximately 10^{-13} moles (Figure 2).

REFERENCES

1. Sweeley, C. C., Elliott, W. H., Fries, I., and Ryhage, R. Mass spectrometric determination of unresolved components in gas chromatographic effluents. *Anal. Chem.* 38, 1549-1553 (1966).
2. Hammar, C.-G., Holmstedt, B., and Ryhage, R. Mass fragmentography. Identification of chlorpromazine and its metabolites in human blood by a new method. *Anal. Biochem.* 25, 532-548 (1968).
3. Samuelsson, B., Hamberg, M., and Sweeley, C. C. Quantitative gas chromatography of prostaglandin E at the nanogram level: use of deuterated carrier and multiple-ion analyzer. *Anal. Biochem.* 38, 301-304 (1970).
4. Gaffney, T. E., Hammar, C.-G., Holmstedt, B., and McMahon, R. E. Ion specific detection of internal standards labeled with stable isotopes. *Anal. Chem.* 43, 307-310 (1971).
5. Wiesendanger, H. U. D. and Kneen, W. A programmed mass spectrometer system providing simultaneous readout of several gases. Proceedings of Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Cleveland, Ohio, March 1969.
6. Hammar, C.-G. and Hessling, R. Novel peak matching technique by means of a new and combined multiple ion detector-peak matcher device. *Anal. Chem.* 43, 298-306 (1971).
7. Klein, P. D., Haumann, J. R., and Eisler, W. J. Instrument design considerations and clinical applications of stable isotope analysis. *Clin. Chem.* 17, 735-739 (1971).
8. Jenden, D. J. and Campbell, L. B. Measurement of choline esters. In: *Analysis of Biogenic Amines and Their Related Enzymes*. Ed. D. Glick, John Wiley & Sons, 1971, pp. 183-216.
9. Hammar, C.-G., Hanin, I., Holmstedt, B., Kitz, R. J., Jenden, D. J., and Karlén, B. Identification of acetylcholine in fresh rat brain by combined gas chromatography-mass spectrometry. *Nature* 220, 915-917 (1968).

Figure 1. Block diagram of multiplexer/demultiplexer for QUAD quadrupole mass spectrometer.

Figure 2 .

DISCUSSION

Dr. Watson: In your recording the total sample was 2 or 3 picomoles?

Dr. Jenden: Yes.

Dr. Watson: 100 ng?

Dr. Jenden: About 400 pg.

Dr. Watson: Are those retracings?

Dr. Jenden: No; that is a raw record of a fraction.

Dr. Watson: How fast are you switching?

Dr. Jenden: This is a multiplexer and demultiplexer. It is essentially a clock which drives simultaneously two sets of semiconductor switches. One of them switches different voltages into the programming book and the other switches the electrometer output into eight different outmoles, so you have eight different smooth, independent channels available.

Dr. Watson: Is it proper to ask you what switching frequency is between those two?

Dr. Jenden: The clock operates at about 100 hertz, so each channel is looked at about ten times a second, and then you use an analog to smooth the circuit.

Dr. Dayton: I want to raise two examples where isotopes like carbon-13 would be useful in man. In the classical biochemical study of biosynthesis, if you start with a simple building block you only need one tracer, but if you want to start in the middle you have the problem that the metabolite can go either of two ways. It can revert back as well as go forward. So, at this point double isotopes are extremely valuable--for example, carbon-14 and carbon-13.

Another point where double isotopes would be very important in clinical pharmacology exists when you study pool sizes because you could introduce one tracer at one point, have it mixed, then introduce the second tracer, and study the mixing of the pools.

Chairman Bush: The second tracer being in the metabolite.

Dr. Dayton: Or whatever. I think these are general examples. Of course I don't see this being done in normal people, but in disease states, such as cancer, where we don't know the penetration of cancer drugs into solid tumors, I think it is really justified.

Chairman Bush: Dr. Fales, do you have any comments?

Dr. Fales: Do stable isotopes have a place in clinical pharmacology? Unquestionably--deuterium is being utilized even today in a very effective fashion. This and the other isotopes are needed even more for their utility as probes of molecular structure and molecular interactions in chemistry, enzymology, and biophysics.

But much has been made in this conference of the possible use of carbon-13, particularly with regard to its use as a substitute for carbon-14. Is this a reasonable hope? I don't think so, and for reasons that should be obvious to everyone here. But the point is that carbon-14 is itself no longer as essential in certain aspects of clinical pharmacology as it used to be. We have new tools. Dr. C. Sweeley's AVA system described by Dr. Gaffney is just one of the most recent tools which prove this point. Using this device we can use a drug's fragmentation pattern to serve as its own tracer in biological fluids. Isotopic substitution of any sort may greatly facilitate the process, as Tom has shown, by serving as a marker for discriminating against background. Deuterium bound to carbon is excellent in this regard since it is easy to incorporate and often sufficiently stable biologically. Nitrogen-15 is an obvious choice for drugs since it is seldom lost in metabolism. On the other hand if we had easy access to carbon-13 intermediates, they would add another useful dimension to such studies since a carbon atom may be even more biologically stable than deuterium. And, as with the other isotopes, if it is lost during metabolism, this fact conveys structural information about the metabolic process. I think that all I am asking is that we allocate carbon-13 to its rightful place among the other stable isotopes. If we try to pretend that it can serve as a tracer to replace carbon-14, thus endowing it with properties it doesn't have, we are likely to have a lot of disappointed pharmacologists bad-mouthing all stable isotopes. That event may set us back in our efforts to make carbon-13 and other isotopes widely used by the whole scientific community.

With this warning we might think about what we need in the future. Obviously, carbon-13 must be made available in more varied forms so that preliminary experiments can be gotten underway. One successful experiment with carbon-13 playing a unique role would accomplish more than all of our speculations here today.

While we need to improve the sensitivity and reproducibility of carbon-13 sensing devices - notably the mass spectrometer and its accessories - what really seems to be required is the construction of a less sophisticated and less expensive isotope ratio mass spectro-

meter capable of making such measurements at high mass. Such an instrument should be designed with isotope ratio analysis in mind from its conception, sacrificing parameters where necessary in the interest of accuracy.

NMR instrumentation needs to be improved in regard to sensitivity if clinical measurements are anticipated. Ideally, NMR needs to be tied to a separation device in the same fashion as GC-MS. I'm not worried about whether this will be done; there is sufficient external pressure right now by related fields to bring this about.

Overall, I think the prospects for utilization of heavy isotopes such as carbon-13 are very bright indeed, so long as, in our enthusiasm, we do not oversell the method. Particularly to be regretted are experiments using carbon-13 as a stunt where other cheaper isotopes would serve equally well.

Dr. M. C. Horning: I don't look at these problems so much from the point of developing instruments, because I am really not very good at that. I use them, and fortunately the other half of our laboratory does most of the development. I am interested in clinical applications, and I agree that the best thing that could be done to expedite the use of carbon-13 would be some good, concrete examples of their clinical applications.

The big advantage of carbon-13 is that it will allow us to investigate things that are just not possible with any other technique. You can think of two very simple examples, one being all types of metabolic experiments or drug studies which one could call the fetal-placental unit. There is no other way of approaching this kind of study.

I see no reason why one cannot easily get permission to use the stable isotopes and use these in cases of legal abortion. This would give one the opportunity to study the transfer of drugs or natural compounds or hormones, and to do a tremendous amount of work studying a field that has essentially been almost neglected.

The other application, which I think is very obvious, is that you can take new drugs, after very preliminary animal toxicity studies, directly to the human. As Dr. Gaffney showed, a one-shot administration of a new drug, labeled with carbon-13, at a level which could not possibly cause any trouble, and produces a tremendous amount of information about distribution, rates of excretion, metabolites, and so forth with this technique. These are just two examples of use that would really expedite the development of this whole field, which I think is absolutely essential.

The other thing you can do with stable isotopes (which I think, from a clinical chemistry standpoint, is very interesting), is to use them as Dr. DeGrazia has done, in developing dynamic tests. Static tests are not that useful, and a challenge test or dynamic test which

will give rates or a profile response of the individual would be very much better. He has already shown that all you need is a simple mass spectrometer where you can do ratios of CO_2 to $^{13}\text{CO}_2$. Just monitoring CO_2 under the appropriate conditions one will be able to evaluate innumerable metabolic pathways, and also metabolism of many drugs.

I think we are all convinced that carbon-13 is going to be very useful, and what we really must do now is actually provide the professional people with concrete examples. This is the most useful thing we could do.

Chairman Bush: Dr. Weiger has some things to tell us about making these isotopes available.

Dr. Weiger: The National Institute of General Medical Sciences through the Pharmacology-Toxicology Program supports a wide and diverse type of pharmacology-toxicology research primarily at medical schools through center and Program Project grants. We have an in-house direct operational component of the Pharmacology-Toxicology Program supported primarily through research contracts. Because of increasing requests from investigators--that is, grantees--for carbon-13 drugs and other carbon-13 compounds, I thought I would mention just one aspect of what NIGMS is doing with their own direct operational research at the Research Triangle Institute, I will try to phrase it or couch it in terms of Dr. Fales' first two priorities.

Dr. Fales says his first priority would be to have much more carbon-13 available in many compounds, and to improve instrumentation useful for the work in stable isotopes. Because of the demand or requests for carbon-13 drugs and the relative difficulty for investigators to get carbon-13 labeled drugs, NIGMS recently entered into a collaborative arrangement with the Division of Biology and Medicine of the AEC to supply NIGMS with certain simple carbon-13 labeled compounds which will be highly enriched. These will then be synthesized into drugs through our direct operational program.

Some notion of what this entails is given by the fact that during the current year we plan to obtain approximately 0.5 kg of carbon-13 in the form of 90% carbon-13 acetic acid, carbon dioxide, urea, acetylene, and other compounds as mutually agreed upon. This has just started. The first delivery of the compounds by Dr. Don Ott of Los Alamos was made to NIGMS-Research Triangle Institute this week, and I hope we will be able to work on the synthetic problems involved in producing the drugs. Some of the examples of drugs that are high on the priority list--and we certainly would welcome comments from others that are in need of these drugs and chemicals--are steroids containing four carbon-13 atoms, dilantin, and barbiturates. Other drugs naturally will be produced as the need arises and as the facility and availability of the material becomes more and more apparent.

This again is a direct operational activity of NIGMS. The Research Triangle Institute is doing this in collaboration with us and in direct collaboration with their grantees. However, the Research Triangle

Institute is a nonprofit organization unto itself, and it may wish to deal with individuals on a private basis. Dr. Monroe Wall is here, and you may wish to talk to him about activities of a private nature not funded through NIGMS.

Chairman Bush: We will continue the formal program. Dr. Klein, do you have a few words to say?

Dr. Klein: I would like to come back to the matter of priorities and development. In January of this year at a meeting in Los Alamos Don Ott, Don Borg, Walton Shreeve, Bert Tolbert, and I were very concerned as to what one can do to augment the use of the stable isotopes. As I have said to many of you on several occasions, it is very much of a bootstrap operation because you need instrumentation, stable compounds, and applications as we have heard again today.

In terms of specifics, the production of carbon-13 and its availability to non-AEC researchers is now made possible through NIGMS. The techniques, the circuitry, or in fact construction of any of the components which we have are available to any one who is interested. Dr. Wall has already contracted to buy this type of circuitry for his LKB.

We have excellent electronic capability at Argonne, and the operation of the Electronics Division as a zero budget, namely as a nonprofit division, means that these things can be sold directly to other non-profit agencies that may wish to purchase them. We are not in any competition with private industry because there is no manufacturer currently making this circuitry. I did want to make clear that it is not necessary for you to develop your own circuits. If you need to do something along this line we can have it designed or can build it for you, at a reasonable cost.

With respect to the clinical instrumentation, in this year's budget the AEC has put a small amount of money (for which we are grateful) toward the development of what was originally envisaged as a clinical breath analyzer. The specifications were simply that it should be able to do very high dilutions of respiratory CO₂ and be very inexpensive. The construction of this prototype is under way.

I think we should have a working version by the first of September 1972, and it is quite likely that if this is of interest to specific people we can arrange either to make the design or to construct one for your use if you are interested.

These are the things that are under way which will provide assistance to you who are interested in doing these kinds of analyses. I would further say that if, individually you have an interest and want to develop it, and we can be of assistance in doing a trial analysis so you can arrange to see whether or not it is of interest to you, we will be very happy to collaborate with you.

Dr. Borg: Can you give us the outlook for that clinical carbon-13 breath analyzer? What is the cost range, and what kind of dilution do you think it will be able to detect?

Dr. Klein: I anticipate that from the standpoint of CO_2 it should go to a dilution of 1 part in 10^6 . It should be a quite compact instrument and hopefully, cost less than \$20,000.

Dr. Sweeley: In so far as mass spectrometry is used as an analytical tool for measurement of stable isotopes, I think there are some complex considerations for the manufacture of compound which need to be brought out at this meeting, especially since General Medical Sciences and the Atomic Energy Commission are becoming increasingly able and willing to produce these compounds for utilization in pharmacology and other areas.

What I am talking about is the problem of where to introduce the label in the molecule. This has got to be done with full cognizance of the mass spectrum of a compound that is to be sold, the metabolites of that compound, the derivatives that might be used for GC mass spectrometer, and many other factors. The choice of where the isotope is put in other words has got to be metabolism-oriented.

If I were going to look at the production of pregnenolone from cholesterol it would be foolish indeed to buy 25-dideuterocholesterol as a starting material. If I were going to look at operations on the steroid in the A ring it might be unwise to have a labeled molecule where a stable isotope, especially deuterium, is incorporated in that part of the molecule.

The compound has got to be designed from the mass spectrum point of view as well. If the label is put in the wrong part of the molecule there may simply be no ions of high intensity that can be used to measure the stable isotopic abundance. So, one has to look at the mass spectrum before choosing the label and where to put it.

One should consider isotope effects, since these might affect the metabolism of the compound seriously, as we have seen in several cases yesterday and today. We tried to do that in the case of the experiment with 6,6-dideuteroglucose, putting it in the part of the molecule where it would not affect the formation of glycolytic bonds and therefore would likely have a very small isotope effect.

Finally, in particular regard to carbon-13, I should point out problems which might be associated with a low percent of carbon-12 when you are going to do multiple labeling. If 90% purity is used and three or four carbon-13 atoms are incorporated into the molecule, as we have seen, the molecular species which exist are rather horrendous from an operational point of view in mass spectrometer analysis, and I would therefore urge AEC to consider further purification of this. I

understand this is the equilibrium mixture between $^{13}\text{C } ^{16}\text{O}_2$ and $^{12}\text{C } ^{18}\text{O}_2$ in the distillation process, but I also understand it is possible to normalize the oxygen content, go through secondary purification, and perhaps increase this to the level of 99%. I think this is urgently needed when multiple incorporation of carbon-13 in the complex molecule is to be considered.

Merck, in Montreal, has been urging me for about 5 years to tell them what compounds to make with deuterium or oxygen-17 labeling, and I said, "It is impossible to tell you this." You have got to go to the man who has looked at the mass spectrum and ask him where a logical choice might be, considering the types of biochemistry or pharmacology that are going to be carried out.

So, I leave this note, that we have got to proceed cautiously and logically in the choice of compounds that are made in these early days, otherwise we might have a shelf full of compounds that are useless to the investigator.

Dr. Clark: I would like to make two comments. As Director of the NIGMS Pharmacology-Toxicology Laboratory, I am interested first in the drug aspect. The collaboration program is exploratory as to feasibility. We hope to clarify the working relationships with AEC, to explore its synthetic avenues, to explore some analytical aspects, and to explore some clinical opportunities based on this to determine for ourselves, at least, costs, scope, and future amount of justifiable effort we want to put in this area.

I also would point out that this represents only one aspect of NIGMS efforts. There are general medical sciences, and we have a great deal of interest among the people in metabolism, genetics, genetics chemistry, and so on; so I don't know where this is going.

Chairman Bush: There was a question some time ago by Dr. Wall, and we didn't take time for it. Would Dr. Horning like to respond to it?

Dr. E. C. Horning: I think it would take too long to go into that problem. Essentially what is involved is the question of using the mass spectrum as a selective detector. In principle you can use either an electrical field instrument or a magnetic instrument. So, it is not a question of the principles involved. It is simply a question of the degree to which each instrument is suitable at this time for that purpose. My personal opinion is that most of the mass spectrometers we are using now are badly designed, with outmoded circuits, and they are at least 5 to 10 years behind the times. That holds for all of the instruments.

Dr. Wall: With a chemical ionization source, fragmentation is greatly reduced, so that now the parent ion or the $m+1$ ion source is essentially a base peak. If you have this type of device (and some of the problems Dr. Sweeley has rightfully alluded to), they can greatly alleviate these problems.

My question is: Does it seem at the present time as if a multiple scanning by that type of instrumentation will be able to do something akin to what is being done via the AVA route? Do you have any feeling on that, Dr. Horning?

Dr. E. C. Horning: The answer is yes, I think almost all of the work can be done by chemical ionization techniques, for two reasons. One is that while it is not true that one always sees the so-called MH ion, nevertheless in all of our experience we see simple spectra, not complicated ones. The spectra are extremely simple and this is a profound advantage.

The other thing is that, if you use chemical ionization, it may be that you can avoid ionizing many of the problems. This is the one that Dr. Watson referred to--the fact that with this ion monitor you see everything, all the compounds you don't want to see. One advantage of chemical ionization is that we may be able to choose reactive species. In other words, if another selective step is introduced, then hopefully we will see a totally different sort of mass spectrum. In other words, many of the ions shown with electron impact ionization won't be seen. There are many compounds you don't want to see. At one time we thought it best to see everything, and I am convinced now that it is not advantageous.

Dr. Fales: Recently we looked at about 48 drugs--common commercial drugs--and in 35 of them the P+1 ion is the largest ion in the spectrum, which does give you a good feeling. Certainly, in the other cases, something as simple as loss of water or loss of a simple fragment is the one largest ion. Furthermore, in this method, the isotope ratios are extremely accurate and reproducible, at least according to a preliminary quick survey by us.

The reason for this is the variety of ion-molecule reactions, which are one of the major sources of inaccuracies in isotope ratio measurements, in ordinary mass spectrometry, and when we are dealing with compounds of higher molecular weight with reactive functional groups. In the case of chemical ionization we simply swamp out the whole effect by making all of the ions result from a single type of ion-molecule reactions. Our preliminary evidence is that there is quite an improvement in that regard.

Dr. M. C. Horning: I just want to make one comment. If you use drug metabolites rather than the parent drug, and then make diluents, and this is on an alpha chain, you will not see in general the m+1. The chances are you will see this in large sections, but if you go to metabolites I think the chances are sort of 50:50.

Dr. Fales: The big problem there is that the trimethylsilyl ether which we have found to be so useful, is unfortunately, almost uniquely poor as a derivatizing agent for the chemical ionization process. In this regard you don't see the parent ions.

Dr. M. C. Horning: I think all of this work is going to be done by chemical ionization.

Dr. Wall: I think that is the way of the future.

Dr. M. C. Horning: No question about it.

Dr. Wall: This is what I was trying to get them to bring out. They brainwashed me too, but I think it is important to bring this out, because there may be many people here who are thinking of acquiring instrumentation, and it is an important consideration.

If I might make a comment, I would like to speak further on this procurement problem. In the first place, as Dr. Weiger and Dr. Clark have just told you, Research Triangle Institute, in terms of the synthesis of carbon-13 labeled compounds, is operating directly out of the NIGMS contract. I would like to say that our own interests are primarily involved in what we will be doing under this contract, because it is through this means, and by the cooperative agreement with AEC, that we are getting the carbon-13 at a very favorable price.

We would like to help other people, but we are really not interested in getting into production extensive outside of our NIGMS-supported work.

In terms of mechanics, I would recommend that you, or others who hear about this program one way or another, write directly to Dr. Weiger or Dr. Clark, with a copy of the letter to me, requesting the desired compound, the purpose, and where you would like to have it labeled.

As Dr. Sweeley has just mentioned, there are a lot of problems. Fortunately we have a fairly good mass spectrometry facility at our place, so we will be able to look at a lot of compounds that we make both for our own internal research needs and those of the groups. Priority, of course, will be given to NIGMS-affiliated supportive groups. Those of you who are operating under other state or federal grants, I would expect, will have to try to make your arrangements through NIGMS. This really has not been spelled out. If we had to do it privately I am afraid the price might get prohibitive, because we can't provide carbon-13 at this favorable price nor do we want to get into competition with the companies that are private enterprises.

We are a nonprofit research institute, but hopefully with clearance from NIGMS we will make these compounds. It will have to go via that route, so we will definitely require approval of NIGMS, and then we will make these compounds in appropriate amounts. Obviously this will not be kilograms, but certainly in many cases there will be no problem making 5 or 10 g amounts. We need to get an idea in terms of our own scheduling and priorities. This is why I am anxious to get as many inputs from those of you who are eligible to get help from this program as possible, because it could well be that a number of you may want the same compound. There are obvious advantages in your knowing this.

Dr. Roth: Last night, while talking about this symposium with Dr. Morgan I decided that he had made a few observations which should be presented after which we will close the meeting.

Dr. Morgan: First of all, I think I would like to take this opportunity to express my own personal thanks, and I hope the thanks of all of you, to Dr. Roth and the organizing committee for what has been a most stimulating, enlightening, and enlightened seminar. Perhaps this is an appropriate time to express our appreciation to him. (Applause)

A few words could also be said about my own personal appreciation for the contributions of both the speakers and the discussants at this meeting, not only the formal discussants but all those who have participated in an *ad hoc* fashion. I have found it most stimulating, although somewhat confusing. To some extent I think the confusion may lead to further stimulation; I hope it will.

When Dr. Roth asked me last night to say a few words in summary about this meeting, we had not anticipated the degree to which summarizing elements would come into the discussion this morning, and that has to some extent, or to a very large extent, obviated the need for me to say anything in the way of technical summarization. What I would like to do instead is make just one or two very brief comments which could be labeled "perspective" rather than "summary".

One thing that has emerged very loudly and clearly to me, amidst perhaps some of the confusion of the discussion where conflicting opinions arose, is some of the differences that exist among us as well as some of the similarities. Dr. Freese alluded to this in his discussion this morning and emphasized the need for cooperative effort. This cooperation extends in many directions. It should extend certainly to reaching some sort of understanding and rationalization of the different points of view of the groups that are represented here. It is absolutely clear that social, political, and ethical considerations enter the consciousness of the clinical pharmacologist and medical research man, perhaps to a much greater extent than they ever do to physical research scientists and a full appreciation of the differences in the role of the different types of scientists in society is of prominent importance.

I was interested to hear, this morning, the comments on the need for the clinical pharmacologist and the medical research people to move more rapidly into utilization of techniques that already exist and to apply them to their own particular uses. I think it is necessary that all of these people get together at a very early stage in the game before these techniques and methods have been developed to the point where they have been fixed in character. To a very large extent, limitations that exist on certain types of applications and techniques exist not because they are intrinsic to the system but because the nature of the development has led them in a certain direction which is not particularly appropriate to another field, such as pharmacology.

I meant to say at the very beginning that one complaint I might offer to Dr. Roth is about the name of this seminar. I have a mild objection, I guess I should say, to seminars on "the use of"

something. Attention should be focused on the problem rather than on trying to force a particular application or series of applications on a particular technique, or on a particular thing.

In thinking about a name for the conference or seminar, I came up with one that intrigued me just a little bit, and that is "The Pharmacology and Psychiatry of Perturbed Molecules." (Laughter) After all, what we are talking about in all of these applications is perturbations introduced into a molecule in order to carry out some function, and I think focusing attention on this particular aspect of the whole problem could allow us to get a somewhat different viewpoint of the relationship among these different things.

There are areas in which perhaps one method is best. There are conflicts between them to some extent, but there is a much greater complementary character to the whole problem, and we should emphasize always, as I said yesterday, the supplemental character that each of these techniques gives to the other. If we focus attention on that perhaps we will reach some sort of new era, shall we say, in the whole field.

Sitting and listening to you people here in the last day and a half, it appears that clinical pharmacology is entering a really new era. You are ridding yourselves of what physical scientists have long called the "black box syndrome." I think you have to interact with the system. This calls for a degree of expertise in certain areas that many of you don't have, but you also bring an expertise to the area that other people don't have. It is very important that these things come together.

In talking about the details of the components of systems, much attention has been paid to the hardware, to the software, to the degree of interaction between those things, and not enough attention (although everyone recognizes it) has been paid to the contribution of the people who are doing it. One of the most important elements in the proper analysis of these systems is in fact the creative character that the operator brings to it and that the scientist brings to his problem.

You have heard of techniques that are highly sophisticated based on exceedingly sophisticated concepts, and to fully understand these and the implications in a particular area is difficult indeed unless there is fully cooperative effort, and by that I mean cooperating at all levels with the people who are involved in it.

Finally, with a type of summary which is perhaps appropriate at this time, I would like to ask what the purposes of a seminar of this type should be. This is my personal opinion:

First, it should bring people together. This conference has succeeded admirably in this respect.

Secondly, to suggest avenues of approach to new or previously imponderable problems. We have made a few starts in that direction. They are more suggestive perhaps than they are fully developed, but it will stimulate ideas.

Thirdly, to clarify the bases of potential interactions. To some degree this has been achieved.

Fourthly, to permit mutual consideration of conflicting demands and requirements. This has been achieved to a large degree. It is impossible at the moment to assess the success of this seminar. That will have to depend on what happens because of it.

I hope that many of you will have made new friends and new contacts which will lead to fruitful collaboration in the future. (Applause)

CONCLUDING REMARKS

The isolation of deuterium by Harold Urey opened a world of discovery to the biologist. This stable isotope was quickly exploited by the late Dr. Schoenheimer at Columbia University in a startling series of tracer experiments which established the concept of dynamic equilibrium. A new era in biological research had begun based on Mass Spectrometric Analysis. A decade later the cyclotron and the atomic pile dramatically focused attention on radioactive isotopes such as carbon-14 and tritium which have since played a dominant but not exclusive role in biological research. Deuterium, the heavy stable isotope of hydrogen, never lost its special place, however, in what must have seemed to many to be a race between the tortoise and the hare.

The decision to develop a facility at Los Alamos for substantial production of the stable isotopes of carbon, nitrogen, oxygen, and sulphur is a significant and welcome event for the clinical investigator. The wisdom reflected in that decision will now certainly be taxed to sustain the challenge inherent in it because that decision also calls for stimulation of other processes, such as the labeling of compounds and the sharpening and simplifying of the technology, as well as development of a thoughtful process for the reasonable yet realistic regulation of the stable isotopes in human subjects.

We know intuitively that a non-radioactive isotope is generally preferred to a radioactive isotope in clinical investigations unless it is apparent that the advantages to be gained from the use of a radioactive isotope justifies the risks. The case for stable isotopes in Clinical Pharmacology and Experimental Medicine is overwhelming.

The organizing committee is grateful to you for coming and for the many lively discussions, both recorded and unrecorded, which took place here. It has been a stimulating and rewarding two days.

Lloyd J. Roth

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November 10-11, 1971, The University of Chicago

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